

MICROSCOPIC FIELDS OF PATHOGENIC ORGANISMS AS PREPARED
FOR LABORATORY DIAGNOSIS ($\times 900$)

1, diphtheria, throat culture; 2, Vincent's angina, throat smear; 3, tuberculosis sputum; 4, pneumonia sputum; 5, gonorrhea smear; 6, rabies smear, brain of dog

A TEXTBOOK OF
BACTERIOLOGY
AND ITS APPLICATIONS

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Revised Edition

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PREFACE

With very few exceptions the textbooks in bacteriology deal chiefly with the medical aspects of the science. While the early achievements and the most important applications of bacteriology have been in the field of medicine, yet, as the subject and its literature have expanded, the various other branches of the science have become of increasing interest and importance.

The original plan and scope of the text has not been altered by the present revision. It attempts to present within a limited compass the fundamental concepts of bacteriology and its most important practical applications, such as might be readily covered within the usual college course. The comments of many who have used this book for various types of students, as well as the author's experience with it as a text for several hundred nurses and students of home economics and of social work, afford convincing evidence that the broad, general treatment of bacteriology is the best introduction to the subject.

Bacteriology, however, is a growing, changing science. Having attained the dignity of a separate science only about seventy-five years ago, it is not surprising that new discoveries of fundamental importance are still of frequent occurrence, and old ideas have to be abandoned in the light of newer knowledge. Recent years have given us rather revolutionary discoveries regarding such matters as bacterial variation, life cycles of bacteria, and the chemistry of bacteria as affecting their antigenic properties. All along the scientific front of bacteriology, advances have been made.

This textbook has been thoroughly revised by making liberal deletions and additions to bring it strictly up to date. Certain chapters have been largely rewritten, as, for example, "The Yeasts" and "The Public-Health Laboratory." Sections have

also been added, "Life cycles of bacteria," "How bacteria cause disease," "Bacteriophage," "The acute food-infection group," and many others. The chapter on classification has been completely revised to conform with the latest "Manual of Determinative Bacteriology," and the appendices "Standard Methods for the Examination of Water" and "Standard Methods of Milk Analysis" have been made to conform to the most recent recommendations of the American Public Health Association.

The author wishes to express his thanks to all who have assisted him in the production of this book. Mrs. Pauline Hitchcock Foster rendered most valuable assistance in the preparation of the original text. Professor Samuel C. Prescott examined the original manuscript with unstinting care, and his many suggestions have been of very great value. Professor Gorham Harris and others have also made valuable constructive criticisms. Last, but not least, I am indebted to my dear wife for her careful reading of the manuscript and proof.

We gratefully acknowledge our indebtedness to the following sources for cuts and prints not specifically acknowledged in the text: the Fisher Scientific Co., Pittsburgh, Pennsylvania; the Scientific Bureau of the Bausch & Lomb Optical Company, Rochester, New York; the American Museum of Natural History, New York City; H. P. Hood & Sons, Boston, Massachusetts; Raymond Pearl, "The Biology of Population Growth," Alfred A. Knopf, Publisher, New York City; and the Yale Medical Press, New Haven, Connecticut.

We have attempted to give due credit either here or in the text for all specific references and cuts used, but material that is a part of the heritage we all possess from sources that are constantly consulted has unavoidably crept in, and it has been impossible to acknowledge this in detail.

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INTRODUCTION

The history of bacteriology is of unique interest to the student of the natural sciences, and illustrates in the most striking manner how the isolated and abstract observations of investigators in rather remote fields may be classified, related, and welded into a concrete and well-defined science of far-reaching application.

The scattered facts and the hypotheses brought to light by the curiosity of Leeuwenhoek, the philosophical speculations and controversial experimentations of Needham and Spallanzani and their followers, the systematism of Müller, and the practical genius of Appert and Saddington in a sense prepared the way for the broader vision of the master mind of Pasteur, through whose painstaking skill and unremitting industry in research in many fields the loose blocks were cemented into a solid foundation for a new science destined to be of wide application to human welfare. From his work on fermentations and on the diseases of the lower animals came the guiding principles of bacteriological investigation. His work, and that of Koch and Lister and their followers, profoundly affected not only the history of a science but the history of human welfare.

It is not surprising that in the early development of the science the greatest forward steps should have been taken in the field of medicine, the study of microbes in relation to disease, for no other phase of the subject is so closely related to the well-being of mankind. But the principles firmly established by the great Pasteur were so wide in their application that from them have come the extension of bacteriology into many other practical fields, as in agriculture, sanitation, food preservation and manufacture, and in numerous applications

of industrial significance. The student to whom these interesting phases of bacteriology are presented cannot fail to gain a new and broader conception of the phenomena of nature, and a truer sense of the relation of microbes to human affairs.

Some acquaintance with the activities of the more common forms of microbic life has become a desirable, and in some cases an essential, part of the professional training for many of the careers of usefulness now open to the aspiring student. Not only for those intending to make a life career in medicine or in the teaching of biological sciences or biochemistry, but equally for those engaging in nursing, household economics, public-health work in its various forms, and in other branches of social service, knowledge of microorganisms is of constant applicability and usefulness.

Especially to the student of household economics, whose training must perforce encompass a clear understanding of the principles of sanitation as well as of the preparation, control, and preservation of food, bacteriology, in its broad aspects, is a subject of fundamental importance and should include not only the bacteria but other nearly related organisms, the yeasts and molds, which may be studied by similar methods. The production of a useful textbook for such students is no mere matter of compilation of a mass of data, but requires careful selection, accuracy of statement, balance, and a digestion of the material to be presented so that it may readily become a part of the professional working capital of the student.

Bacteriology is essentially a science in which laboratory experimentation and research are desirable. It is understood that a well-organized and comprehensive series of laboratory exercises is to accompany the use of this volume, and will serve to bring into the personal experience of the student the actual demonstration of many of the principles and processes which are here so admirably portrayed.

Professor Hilliard has put into this work not only the enthusiasm of a lover of his science but the results of a long and successful experience as a teacher. He has made a wise selection of material, an accurate and well-balanced presentation.

of the most significant aspects of bacteriology for the type of student which he aims to instruct, and has done it with such clearness of statement and excellence of diction, in short, with such directness and readability, that the book should be useful to the lay reader who seeks to broaden his biological knowledge as well as to the student to whom it is a part of the professional scientific equipment.

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A TEXTBOOK OF BACTERIOLOGY AND ITS APPLICATIONS

CHAPTER I

INTRODUCTION. HISTORY OF MICROBIOLOGY

"It may be said that it is thanks to them (bacteria) that the continuation of life is possible on the surface of the globe."¹

We are more certain of the truth of this utterance now than when it was written, for with the growth of our knowledge of the world of the infinitely small living organisms we are prone to attach ever greater importance to their contribution to conditions of existence. The relation which bacteria bear to disease is well appreciated, but the manifold rôles which they play in relation to food supply, the arts, and industries are not commonly known.

Is it not surprising, if the introductory statement is true, that the significance of microorganisms — in fact, any real knowledge of their existence — was almost completely overlooked until the middle of the last century? There are men living today who can clearly recall the wonderment and skepticism accompanying the early reports on the relation of bacteria to fermentation and disease; and is it too much to say that even now there are people who "don't believe in germs"?

The dawn of our knowledge of microorganisms awaited patiently the development of the physical sciences, particularly optics, just as now much fundamental knowledge must needs await the painful toil of physics, chemistry, and biology before it can create new industries or relieve the travail of mankind in some way. A new instrument turned on an old

¹ A. Magnin, *Bacteria*, translated by Sternberg. 1880.

problem may any day change our philosophy or our activities, just as magnifying lenses, placed in series (compound microscope), did when focused upon rain water and putrefying substances.

The compound microscope. Hans Janssen (Jenssens) (1590), a weaver, seems to have been the earliest user of compound lenses. They were first seriously applied to biological subjects

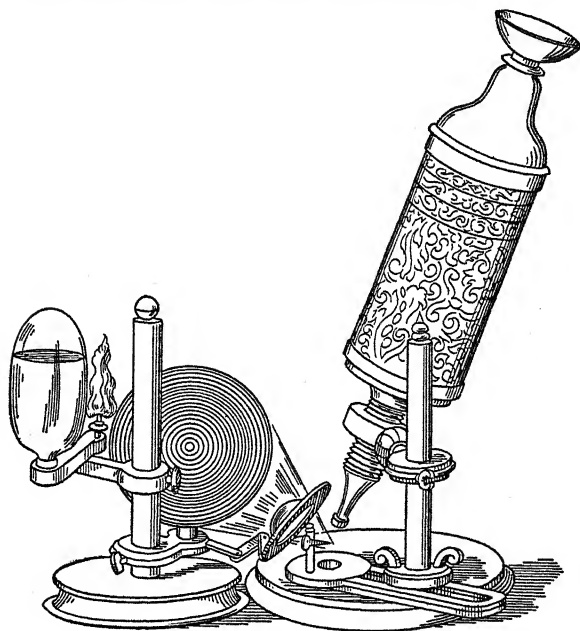


FIG. 1. Robert Hooke's microscope (1665)

in the middle of the seventeenth century by Hooke, Malpighi, Leeuwenhoek, and others. The early microscopes were very crude affairs, giving comparatively low magnifications, and were focused by pushing and pulling the drawtube in and out. The patience necessary to get an object in focus by this method can scarcely be imagined by students today, with the beautiful mechanisms that are put into their hands in well-equipped laboratories. With the take-it-for-granted attitude of the present-day student, who can start where science left

off yesterday, it seems sometimes as though it would be almost worth while if he had to commence with the crude devices of the pioneers. It would sharpen ingenuity and create a more wholesome respect for the labors of the legions of workers who have made present-day classroom equipments possible.

Since the conception of placing lenses in series, four fundamental principles have been introduced into the microscope mechanism. (1) Dolland, in 1844, introduced the immersion lens which provides a homogeneous refraction system for the light as it passes from below up through the lenses. A study of the diagram (Fig. 3) will make this plain. Clear pictures at much higher magnifications are made possible. (2) Abbé, about 1870, introduced the substage condenser now bearing his name, which makes possible more brilliant illumination of the microscopic field. (3) Siedentopf and Zsigmondy, about 1903, introduced the indirect, or horizontal, method of illumination, which makes visible

minute and especially the less refractive objects which appear luminous on a dark background. Many organisms and objects, otherwise invisible, may be studied with this dark-field illumination or ultramicroscope. (4) Barnard has extended the limits of microscopy by the use of the short, invisible ultra-violet light waves produced by the quartz mercury-vapor lamp. Since ultra-violet light does not pass through glass, quartz condensers

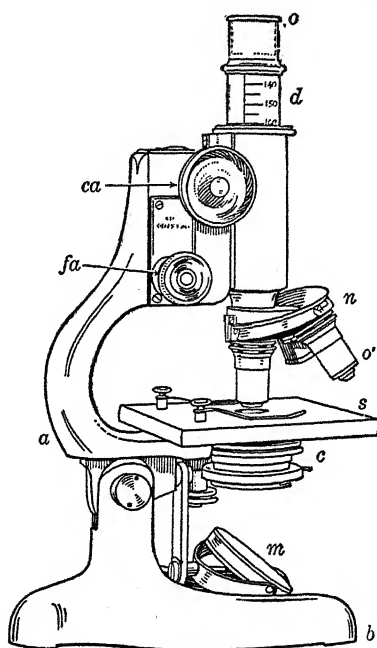


FIG. 2. A modern microscope

o, ocular; *d*, drawtube; *ca*, coarse adjustment; *fa*, fine adjustment; *n*, nose piece; *o'*, objective; *s*, stage; *a*, arm; *c*, condenser; *m*, mirror; *b*, base

and lenses must be used. Microphotographs have been obtained using light of $275\ \mu\mu$ wave length, giving 2200 magnification. This invention will surely lead to important discoveries regarding the nature of filterable viruses.

Discovery of bacteria. Before the discovery of the microscope bold philosophers had speculated on the existence of invisible

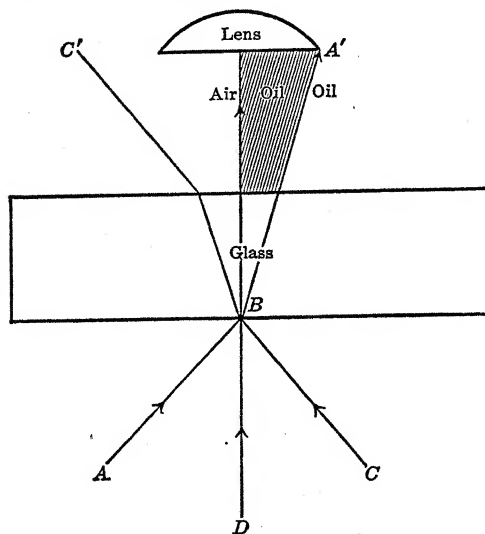


FIG. 3. Diagram illustrating the principle of oil immersion

The ray of light CBC' is bent as it passes from glass into air, while ABA' , passing into cedar oil, which has approximately the same refractive index as glass, continues in a straight path

organisms. Fracas-torius, a Veronese, in a treatise written in 1546, stated the theory of infection by microorganisms. Athanasius Kircher (1602-1680), a Jesuit priest and one of the very earliest of the microscopists, "employed the microscope in investigating the causes of disease" and enunciated the germ theory of disease. The first authentic description of bacteria was given by Anton van Leeuwenhoek (1632-1723), in his

famous letter to the Royal Society of London in 1683. One oft-quoted sentence will bear repeating, for it describes so well the first impression of the amateur with his initial observation of an infusion under the microscope :

I saw with wonder that my material contained many tiny animals which moved about in a most amusing fashion ; the largest of these showed the liveliest and most active motion, moving through the water or saliva as a fish of prey darts through the sea ; they were found everywhere, although not in large numbers.

Though a prodigious worker (he ground his own lenses and is said¹ to have had two hundred and forty-seven complete microscopes) he lacked vision in regard to the significance of his observations, and his discoveries went almost unnoticed.

It was about a century later when Müller revived interest in "these microscopic animals" and described many fundamental structural details. Ehrenberg (1795-1876) courageously attempted the classification of the "Infusoria" (1838), and introduced many of the names which still persist in bacteriological nomenclature. His was the most scholarly work on microorganisms that had been done up to this time, and did much in encouraging system and thoroughness in subsequent work in bacteriology.

Spontaneous generation. The discovery of microscopic organisms did much to keep alive the ancient controversy regarding spontaneous generation. Harvey and Redi had almost silenced the advocates of this now seemingly absurd idea, but a new battle ground was opened by the apparently spontaneous appearance of "infusoria" in all sorts of clear solutions containing organic matter. It is little wonder, now that we know how ubiquitous microorganisms are, and how difficult many of the "seeds" or spores are to kill, that the evidence was for a time baffling. The reader is probably familiar with the development of this historic intellectual contest from other readings;¹ so we shall pass it over, mentioning only Tyndall's ingenious work which practically settled the question for all time, unless it shall sometime be revived seriously by the biological chemist. Tyndall had been doing experiments in physics with what he called "optically pure air," or air absolutely free from floating particles. Through the bottom of the boxes used for these experiments he introduced test tubes. When the air was demonstrated to be "optically pure" he introduced various putrefiable solutions into the test tubes with a long thistle tube, and then sterilized these by immersion in a bath of hot oil. They remained clear and free from life for months. Finally the door to the closed box was opened,

¹ See Locy, *Biology and its Makers*, pp. 277-293.

and outside air laden with dust and germs was admitted for just an instant. Shortly thereafter putrefaction occurred in all the tubes. Seldom if ever has so ingenious an experiment been devised for any purpose.

Pasteur: fermentation and disease. The modern era of bacteriology and the manifold practical applications of the subject

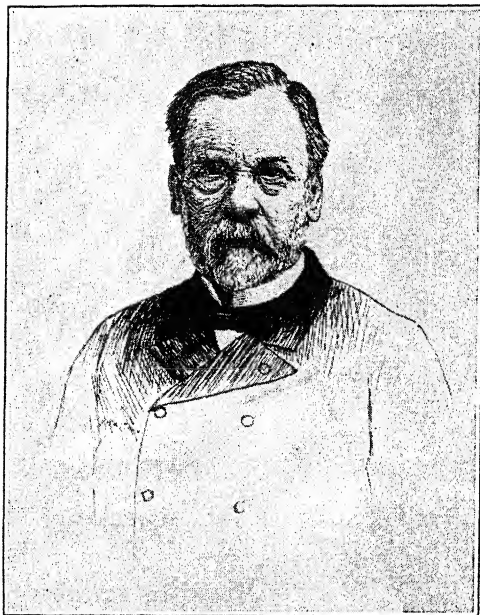


FIG. 4. Louis Pasteur (1822-1895)

date from the work of Pasteur. Louis Pasteur (1822-1895) was born of humble parents in Dôle, France. He was a normal boy, enjoying fishing and play, but was destined to give to mankind the greatest gift of the ages — the secret to the conquest of many diseases. During his later years of schooling he became greatly interested in chemistry and physics, and gained his early distinction in these fields. While work-

ing on the action of the crystals of certain tartrates upon polarized light he demonstrated that the optical properties depended upon the arrangement of the molecules and not upon their chemical composition, thus opening up the field of molecular physics and physical chemistry. It was also during these studies that Pasteur discovered that fermentation of racemic acid solution destroyed the dextrorotary action. This observation led to an interest in fermentation generally, and from this time on Pasteur's contributions were chiefly in

the field of biology. Numerous and important were Pasteur's works, and so remarkable his character and life that the reader is strongly urged to consult some one of the several excellent biographies. The whole development of modern medicine and sanitation rests upon the fundamental discoveries made by him.

Having thoroughly established the general relation of microörganisms to fermentation, Pasteur saw that certain abnormal changes which occur in wine and beer might also be caused by foreign or undesirable organisms, and, being called by the great brewery interests to study that problem, he soon proved that the "diseased" beverages had organisms present that did not occur in the normal liquors. He also discovered that the undesirable changes could be prevented, without injuring the wine, by applying heat of a lower temperature than boiling. This process, known as pasteurization, has subsequently been applied to other products, notably milk, and is of great practical and sanitary importance.

The silk industries in southern France at this time (1865) were in a distressing condition on account of the ravages of a disease of the silkworms. Pasteur was persuaded to undertake the study of this disease. "In a few hours after his arrival he had already proved the presence of corpuscles in certain worms, and was able to show them to the president and several members of the agricultural committee, who had never seen them. . . ." To prove cause and effect between the corpuscles and the disease, and the method of prevention, was a long and difficult task and consumed five years. When this disease, "pébrine," was conquered, it was discovered that a second disease of silkworms was also present, and he must needs stay on to conquer this.

Pasteur emerged from this work more or less broken in health, and grieved by deaths in his family; but the achievement was of inestimable importance, because it was the first carefully controlled demonstration of the germ nature of a disease in animals, and the establishment of practical and successful methods of combat.

•

Microbes and man. The first application to human disease was made by Joseph Lister, an English surgeon. Suppuration in wounds very much resembled fermentations, or putrefactions in dead organic matter, he reasoned, and therefore both may have a common cause; and if antiseptics will arrest fermentations in flasks or in sewage, their application to wounds may be salutary. Thus originated antiseptic surgery, and later aseptic surgery, to exclude all infectious agents during operations. Operations hitherto dreaded and often fatal on account of such infection, and puerperal fever following childbirth, are now almost things of the past where modern methods are used.

Davaine had discovered the bacillus causing anthrax in cattle and sheep, and had succeeded in inoculating healthy animals with this disease, but his results were questioned because of the belief that the blood of the sick animal carried a "morbific" substance, and that the bacilli were a corollary of the disease rather than the cause. Pasteur and Koch almost simultaneously (1876-1877) and independently proved convincingly that the rods found in the blood of animals sick or dead from anthrax were indeed the true causative agents. Pasteur cultivated the bacilli, carrying them through one hundred transfers from one tube to another, when it would be inconceivable that any trace of the original drop of diseased blood could be present, and still produced the disease in animals by inoculating this final culture.

Vaccination. Perhaps the greatest triumph of bacteriology, and Pasteur's greatest work, was the demonstration that animals could be rendered immune to certain specific diseases by artificial inoculation. To be sure, Edward Jenner had initiated preventive medicine in introducing vaccination against smallpox (1796). This still remains the most important and beneficent single discovery ever made. But Jenner used a weakened virus that exists in nature, and applied it to the art of preventing a loathsome and fatal disease. Pasteur, on the other hand, discovered how a given virus or microbe might be weakened or attenuated, and could then safely be used to establish an immunity in the host.

His original discovery was accidental. He had been working with the chicken-cholera virus, which was highly fatal. Upon returning from a vacation he found these cultures incapable of causing the disease; but, what was more remarkable and important, the fowls inoculated with these old cultures were refractory to subsequent inoculations of virulent cultures which killed normal birds. This unlocked the secret to a rational science of preventive medicine. Pasteur soon succeeded in protecting animals from anthrax by inoculating them with a culture artificially weakened by cultivation at a relatively high temperature. His most celebrated and dramatic experimental demonstrations were given at Pouilly-le-Fort, in 1881. Fifty sheep were put into two pens of twenty-five each. Those in one pen were inoculated with Pasteur's "vaccine," and, after a suitable interval, all fifty sheep were injected with a fresh culture. The protected sheep all lived, as Pasteur had predicted they would, while all in the other pen succumbed to the disease. All skeptics (and there were many to combat this patient, indomitable little French scientist at every step) were forever silenced on this point. This principle was first applied to human beings in the disease rabies, or mad-dog bite. Here was a disease 100 per cent fatal wherever it manifested itself. Pasteur had discovered how this

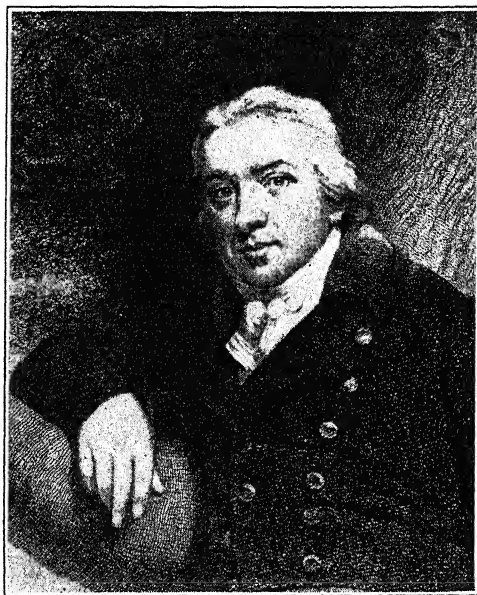


FIG. 5. Sir Edward Jenner (1749-1823)

two pens of twenty-five each. Those in one pen were inoculated with Pasteur's "vaccine," and, after a suitable interval, all fifty sheep were injected with a fresh culture. The protected sheep all lived, as Pasteur had predicted they would, while all in the other pen succumbed to the disease. All skeptics (and there were many to combat this patient, indomitable little French scientist at every step) were forever silenced on this point. This principle was first applied to human beings in the disease rabies, or mad-dog bite. Here was a disease 100 per cent fatal wherever it manifested itself. Pasteur had discovered how this

virus could be changed in virulence by passage through different species of animals, and could be weakened by drying, but he dreaded to try his experiment on a human being for fear the reaction might not be the same as in animals. One day (July, 1885) a shepherd boy, Joseph Meister, was brought in horribly bitten by a mad dog. There was no hope for the boy, and Pasteur consented to administer the treatment. The disease was staved off and the boy lived. The treatment became known as the Pasteur treatment, and shortly afterward the first of the Pasteur Institutes was established in Paris. A much improved, safer, and simpler treatment, using killed instead of attenuated virus, is now almost universally used in this country. Pasteur carried on his remaining work in Paris surrounded by brilliant scholars, as Metchnikoff, Roux, Yersin, and Calmette, who caught the spirit and genius of the master to pass on to this and to coming generations.

Robert Koch. While these epoch-making discoveries were being made by Pasteur a host of investigators were entering the field of bacteriology to perfect the methods of study and develop the various applied fields. The greatest contemporary was Robert Koch, a German physician who started as a rural practitioner. His pure-culture methods, the painstaking microscopic studies of organisms to learn their life cycle, the use of anilin dyes for staining bacteria, and, finally, the use of liquefiable solid culture media for obtaining pure cultures (1881) are contributions in technique which proved of the utmost importance in advancing the knowledge and study of bacteria. They transformed bacteriology into a science, and the modern era may be dated from this time.

The two decades 1880 to 1900 mark the golden age of the science. One great discovery followed another so rapidly that the entire theory and practice of medicine, sanitation, and the fermentation industries were revolutionized, and the application of microbiology to soil fertility and food preservation was greatly advanced.

Koch's methods first bore fruit in his great discovery of the tubercle bacillus as the cause of tuberculosis (1882). At

this time he announced what are known as *Koch's postulates*, which have since served as a guide in proving the germ cause of any disease.

Parasitic protozoa. In 1880 Laveran, a French military surgeon, described a protozoan as the parasitic agent in the blood of malaria patients. While protozoan parasites in man had been described earlier, notably the amoeba causing one form of dysentery (1860), nevertheless this discovery of the malaria parasite was of momentous importance because it led to ultimate conquest of one of man's worst enemies.

In 1873 Loesch described an amoeba with pathogenic properties which he recovered from the human intestine. Some fifteen years later Koch, Gaffky, and others associated this organism, *Endamoeba histolytica*, with one form of dysentery. Amoebic dysentery is not necessarily a tropical disease. An outbreak, traced to infected water at certain hotels, occurred amongst visitors at the Century of Progress Exposition in Chicago during 1933.

The flagellated, free-swimming protozoa known as trypanosomes were first described as parasitic in man by Bruce in 1894 in the dreaded African sleeping sickness. Several parasitic forms of flagellates in animals are now recognized.

In 1905 Fritz Schaudinn made the brilliant discovery of the causative agent of syphilis. The *Treponema pallidum* long eluded scientists, owing to its resistance to ordinary staining methods, and to its invisibility in the unstained condition except when viewed by the special dark-field microscope. In 1910 Ehrlich, after exhaustive research, announced the discovery of the first synthetic specific drug, the famous "606," or salvarsan, which is a cure for syphilis.

Filtrable viruses. Some organisms are so minute that they pass through filters that prevent the passage of bacteria. Such organisms are called filtrable viruses and are usually ultra-microscopic in the sense that they cannot be viewed by the highest-powered microscopes.

In 1898 Löffler and Frosch, working with the lymph of cattle suffering from foot-and-mouth disease, discovered

that the fluid which had been filtered through a bacteria-proof filter retained its infective properties. A microorganism either so small or so plastic as to pass through this filter was present. In this particular disease the organism comes barely within the range of vision of the highest-powered microscope.

Since this discovery many filter passers have been described as the cause of plant, animal, and human diseases. Among the human diseases caused by such viruses are smallpox, rabies, and infantile paralysis. In 1933 Smith and others succeeded in transmitting influenza to ferrets, and later to mice, and demonstrated the cause to be a filtrable virus.¹

Diseases of animals due to such agents include pleuropneumonia of cattle, hog cholera, and apparently chicken sarcoma. In plants the mosaic disease of tobacco and of sugar cane is due to filtrable viruses.

Plant diseases. Plants as well as animals are subject to infectious diseases. Many of the lower fungi, such as the mildews, rusts, and smuts, cause a wide variety of diseases and serious economic losses in crop plants. Bacteria are also the cause of specific diseases in plants. Burrill first described such an infection in the fire blight of apple and pear trees in 1880. Today over forty diseases of plants are ascribed to bacteria, including the wilt disease of cucumber, the brown rot of tomato and potato, the black rot of cabbage, and crown gall in various plants. The work on crown gall by Erwin Smith, whose name is associated with much of the pioneer work in plant pathology, is of special interest, as these galls, or tumors, in plants resemble malignant tumors in animals.

Sanitary science. A knowledge of microbes as the cause of disease gave a rational explanation to the sources of infectious agents and their methods of distribution. The factory system, accompanied by the concentration of population in cities, had resulted disastrously to the public health. Thomas R. Malthus's famous "Essay on the Principle of Population" (1798) had both a sobering and a stimulating effect on his contemporaries. The causes of misery, poverty, and disease

¹ Smith, Andrewes, and Laidlaw, in *Lancet*, July 8, 1933.

were discussed from a statistical standpoint, and there sounded a new social and humane note. Modern vital statistics, so important today as a measure of health conditions and in the study of epidemics, were firmly established by William Farr. The report of Sir Edwin Chadwick on the health of the laboring class (1842), Sir John Simon's Public Health Reports to the Sanitary Commission of London, and Lemuel Shattuck's Report of the Massachusetts Sanitary Commission (1850) carried the conviction that diseases could be limited to a certain extent by rational legislation and public control. The relation of water to the spread of such diseases as typhoid and cholera was convincingly proved by the studies of Dr. William Budd, and by John Snow in his famous study of the London "Broad Street Well" epidemic of cholera (1854). The establishment of a school of public health at the Massachusetts Institute of Technology, under the direction of William T. Sedgwick, to train public-health leaders, and the writings of this great teacher, have had a potent influence in the promotion of the health movement in America.

Although not strictly consisting of discoveries in bacteriology, the history of the public-health development sketched above is the outcome of a knowledge of bacteria.

Two other lines of development must be mentioned — the theories of immunity and our knowledge of insects in relation to the spread of disease.

Immunity. We have mentioned the work of Jenner and Pasteur in preventive medicine. The work of Metchnikoff probably introduced the first sound idea for an *explanation* of immunity. He observed (1884) that certain amoeboid cells in a tiny Crustacean actually devour and absorb certain parasitic yeast cells, and he extended his observations to build up a cellular theory of immunity which was the precursor of our modern theories. In 1886 Theobald Smith performed the pioneer experiment in immunity by demonstrating that immunity from hog cholera can be secured by injecting the filtered products of the causative organism. This led to the remarkable work of Roux and von Behring on tetanus and

diphtheria toxin and to the potential conquest of the latter of these diseases by the use of antitoxin (1894), then to the modern Schick test to detect susceptibility, and finally to immunization by the toxin-antitoxin treatment. Further facts may be obtained from the chronological history in the Appendix.

Insects and disease. T. Smith (with F. L. Kilborne) first proved the transmission of disease through the medium of a blood-sucking parasite in the case of Texas fever in cattle through the cattle tick (1893). Ross demonstrated the transmission of malaria by the *Anopheles* mosquito in 1897, and Walter Reed, with the American Army Medical Commission sent to Cuba in 1900 to study yellow fever, proved this disease to be spread by the mosquito *Aedes*. Since then many diseases, including typhus fever and bubonic plague, have been traced to insects, and these discoveries have led to methods of control. The work on insect *vectors* ranks among the leading discoveries from the standpoint of their value to mankind. Certain dreaded plagues of the race have been stamped out of regions like the Panama Canal Zone, which were hitherto uninhabitable on account of diseases.

The beginning of the twentieth century, as we have seen, found the science of the "infinitely little world" rapidly coming under the dominion of man. Most of the applied fields had been entered. The work of investigation and research has earned a rich reward in this golden age of discovery. Some of the most fatal diseases of mankind have been brought under control through sanitation and preventive medicine. Food in greater abundance and variety has been made possible by more scientific agriculture, controlled conditions of fermentation, and the application of the several arts of preventing food deterioration by microbic action. We live in a cleaner, happier world as the result of an awakened public and social sense of our ability and our responsibility to control these destructive forces of nature.¹

¹In Appendix A will be found a chronological history of the more important discoveries in the science of bacteriology.

CHAPTER II

MICROÖRGANISMS. THE MOLDS

Bacteriology is concerned primarily with unicellular organisms which are classified as bacteria. It is common practice, however, to include a consideration of certain other closely related organisms which belong to either the plant or the animal kingdom, and which require the use of the microscope in their study. Living things of such minute size may be included under the term *microörganisms*, and their science is sometimes called *microbiology* (a synonym for *bacteriology*).

The following outline gives some idea of the relation existing between the bacteria and other plant groups:

Schizophyta (organisms that reproduce by a simple cell division, known as fission)

Schizomycetes (organisms not possessing chlorophyll, that reproduce by fission)

Eubacterales (true bacteria; single cells, never in sheathed filaments)

Thiobacterales (filamentous bacteria; cells containing either sulphur granules or bacteriopurpurin)

Schizophyceæ (organisms possessing chlorophyll, that reproduce by fission)

Thallophyta (plants with undifferentiated roots, stems, and leaves)

Fungi (do not possess chlorophyll)

Myxomycetes (slime molds)

Eumycetes (true fungi; thallus formed of colorless threads)

Phycomycetes (alga-like fungi; reproduce by sporangiospores, and sexually by zygospores)

Ascomycetes (reproduce by conidia, or chain spores, and by ascospores borne in an ascus, or sac)

Saccharomycetes, or yeasts (reproduce by budding or ascospores)

Basidiomycetes (reproduce by basidiospores borne typically on a four-pronged structure, the basidium)

Fungi Imperfecti (a large group not reproducing sexually or in a way characteristic of any of the other groups)

In addition to the well-characterized groups shown in the outline there are certain organisms whose relationships or whose certain identity as living agents are not yet fixed. These include the ultraviruses, or organisms too small to be viewed with the most powerful microscopes that we possess. They represent a very important group of organisms, as several important diseases of plants and animals are caused by them. Also there is a special agent, or substance, called bacteriophage, which resembles the viruses in many essential respects, and which causes a transmissible lysis or dissolution in bacteria.

Bacteriophage is a filtrable agent, passing filters with pores large enough to permit the passage of particles 50 millimicrons (one twentieth of a micron) in diameter. It increases only in the presence, and at the expense, of living bacteria, which it dissolves.

There are two opposing views as to the nature of bacteriophage. One, championed chiefly by D'Herelle, believes it to be a living filtrable virus which is an essential parasite of bacteria. It invades the bacterial cell, reproduces at its expense, and is liberated to invade other bacteria when the cell is ruptured.

The other theory was originally advanced by Bordet. It claims that bacteriophage is a lytic principle derived from the bacterial cell itself, and that the suicidal tendency can be conferred upon other cells. He believes it to be a transmissible, destructive, nonliving enzyme-like agent.

The arguments advanced to support or defend these two theories are not wholly convincing either way. Bacteriophage is *particulate*, is sensitive to chemical and physical influences, is adaptable, and appears to increase or multiply at the expense of living bacteria only. If it is not a living agent, it would appear that we must abandon the idea that so-called filtrable viruses generally are alive. Whatever its nature, its importance in bacteriology is profound, and further understanding and control of phenomena related to bacteriophage are bound to yield important results.

Organisms known as *Rickettsia* are found in a parasitic (or symbiotic) relation in certain insects and higher animals, and have been demonstrated to be the cause of several diseases in man, including typhus fever and Rocky Mountain spotted fever. *Rickettsia* resemble bacteria in their morphology but have not yet been successfully cultivated in artificial media and have different staining properties.

Microörganisms belonging to the animal kingdom which are of most importance belong with the Protozoa, or one-celled forms. Examples are the *Entamæba histolytica*, which causes dysentery, and the *Treponema pallidum*, which causes syphilis.

Microbes and evolution. Both structurally and functionally, microörganisms are the most primitive of all living beings. We may infer, therefore, that in the scale of organic evolution

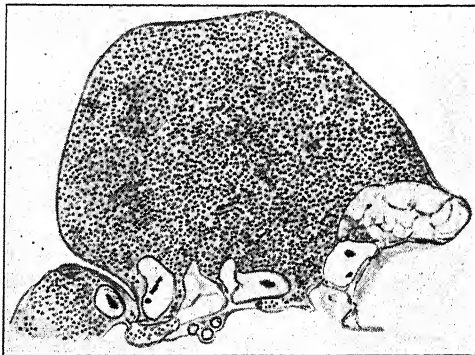


FIG. 6. *Rickettsia* in the stomach epithelium cell of an experimentally infected mouse

Courtesy of S. B. Wolbach

they belong at the bottom, and that forms similar to those now existing represented the first living beings upon the earth. As to proof of their actual antiquity, some hundreds of species of fossil fungi have been found preserved in the tissues of plants fossilized in the rocks of the Carboniferous age,¹ and the diseased bones of fossilized animals give conclusive evidence that parasitic bacteria, probably tubercle bacilli, existed during the same geologic period.

Either the simplest one-celled green algæ or certain bacteria which are independent of other organisms for their food supply might have appeared first. Arrhenius has reasoned that one of the resistant spore-bearing bacteria may have escaped from

¹The strata containing the extensive carbon, or coal, deposits of the earth.

some other remote planet, and, projected through the ether by light pressure, may have survived the vicissitudes of a journey to this earth and started organic life here. The origin of life is, of course, a pure speculation, but in any case we must consider the elementary organisms as playing an important part in the earliest history of organic life and in all that has followed.

Basis of classification. It is the practice in taxonomy, or classification, to base relationships upon visible structural resemblances or differences in individual organisms. With bacteria, because of their minute size, and because bacteria fundamentally different may appear identical under the microscope, this system alone cannot be used. Entirely unique methods, those of culture, or cultivation, and the study of the reaction of masses of organisms upon their food supply, must be resorted to. Apart from the interest which the biologist has in classification, the practical importance of microorganisms depends upon the fermentations, or chemical changes, which they induce, so that here also the desired information is obtained by the culture method. The fermentations or physiological characters depend upon the enzymes, or ferments, which are secreted, and which act upon the food substances in the immediate environment to render them available for passage through the cell membrane or wall. This property is as characteristic and as constant as is a morphological character. The preparation of culture media and the planting of microorganisms in this food material constitutes an important and special technique that has been developed by microbiologists.

The molds. The word *mold* is used to designate certain fungi commonly met by the microbiologist. It has no exact meaning, any more than *germ* has, but serves a useful purpose. There is no sharp line of cleavage between molds, bacteria, yeasts, and higher fungi. There are connecting links, or border forms, both among the higher and lower colorless plants.

True molds have a plant body differentiated into vegetative and reproductive elements. The plant body is sufficiently large and differentiated so that the morphology can be used,

as the basis of description and classification. The vegetative, or assimilative, part of molds is composed of a web of threads called the mycelium. The separate threads, or *hyphæ* (singular, *hypha*), branch in a complex manner. The hyphæ may be one-celled, containing many nuclei, or they may be broken into cylinders by the formation of cross walls called *septa* (singular, *septum*), when the individual cells may be unicellular or multicellular. Cells containing more than one nucleus are called *cœnocytes*. Growth of the hyphæ may occur by division of the cells in any part of their length (intercalary growth), or only the tip cells may divide (apical growth). Practically always septa are formed perpendicular to the long axis of the hypha, so that this threadlike structure results instead of a solid mass of tissue growth.

The mycelium may penetrate into the substratum upon which it is growing, but the visible part is aërial. The mycelium derives the nourishment for the mold from organic matter in the substratum. The aërial portion serves to spread the mold over the surface, and also forms specialized erect hyphæ (fertile hyphæ) for the purpose of reproduction. The methods of forming reproductive cells, or spores, are very diverse and constitute an important characteristic of the molds and serve as a basis for identification and classification. Most, if not all, molds, in addition to forming these differentiated asexual spores, also reproduce by a sexual process, consisting of the fusion of two cells and their nuclei to form reproductive bodies. While the type of sexual reproduction must often be taken into consideration for specific classification, still it is possible with the more common molds to depend for this purpose upon the type of asexual spores formed.

We shall confine ourselves to the description of two common molds to give a general idea of the two principal classes, the *Phycomycetes* and the *Ascomycetes*. A key for the identification of some of the common genera and species is appended to this chapter.

Rhizopus. One of the most widely disseminated of the *Phycomycetes* is *Rhizopus nigricans*, the common black bread

mold. If bread is dampened and kept in a moist, warm place for a few days, almost always there will appear upon it a fluffy, white, cottonlike growth with clumps of little black balls interspersed. This is probably, though not necessarily, *Rhizopus*. If a closer inspection shows the black balls, which are the fruit, or spore-bearing heads, to be borne in a whorl

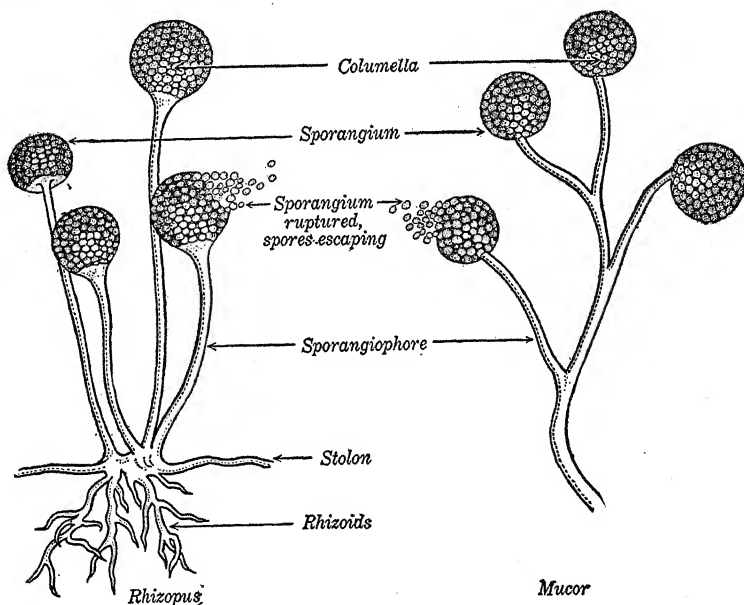


FIG. 7. Diagram of *Rhizopus* and *Mucor*

The columella is indicated by the less dense region in the lower part of each sporangium

or cluster, and if threads connect the successive adjacent clusters, then the identification of *Rhizopus* is complete. The fluffy growth is the mycelium composed of individual hyphæ; the spore heads are sporangia borne upon special erect aërial hyphæ known as sporangiophores; and the hyphæ connecting the clusters are stolons which may be compared to the "runners" of strawberry plants.

If some of the *Rhizopus* is now carefully teased away from the bread and mounted under the microscope, we find the

variously branching hyphæ in a young culture to be nearly colorless. The threads are filled with a slightly granular cytoplasm containing nuclei, and sometimes with vacuoles. Search for cross walls, or septa, does not reveal any.

Turning to the sporangiophores, we find them straight, unbranched, with the walls thickened and often darkened or smoky in color. The contents show no special structure. Where the several sporangiophores arise in a whorl are seen some interesting, tortuous hyphæ, the rhizoids, or rootlike processes, which anchor the mold to the surface.

At the extremities of the sporangiophores are the sporangia, each containing many hundreds of spores. The intact sporangium appears like a dense, black, nearly spherical ball. The thick-walled black spores give it its color. It may appear lighter in color directly above the sporangiophore in a hemisphere reaching about to the center of the sporangium. This appearance is due to an internal structure which is revealed when we examine a broken sporangium.

Old sporangia are very fragile, and the wall of the sac bursts at the slightest provocation. In the microscopic mount they appear like irregular black masses composed of the spores streaming out. The spores vary in size; the sides are often slightly flattened. At the base of a broken sporangium are seen fragments of the frayed outer sac, and a persisting hollow dome. This is the columella, the function of which is to press upward from the inside to break the sac if no extraneous force ruptures it.

We may turn now to the development of the sporangium. An erect, fertile hypha arises from the attachment of the stolon. The end of the hypha becomes swollen and more granular, the cytoplasm at the periphery being more dense and containing many actively dividing nuclei. Vacuoles appear in a hemisphere dividing the denser outer protoplasm from the central portion, and a double cell wall, cleft at the juncture with the outer sac wall at the base, is formed. The nuclei at the periphery arrange themselves more or less symmetrically, and cell walls are formed around them. These

cell walls gradually thicken, become dry, and draw apart, forming the separate spores.

The spores, upon germinating, give rise to mold plants identical with the parent plant, completing the cycle.

In addition to the asexual method of reproduction just described, *Rhizopus* also forms special thick-walled resting spores

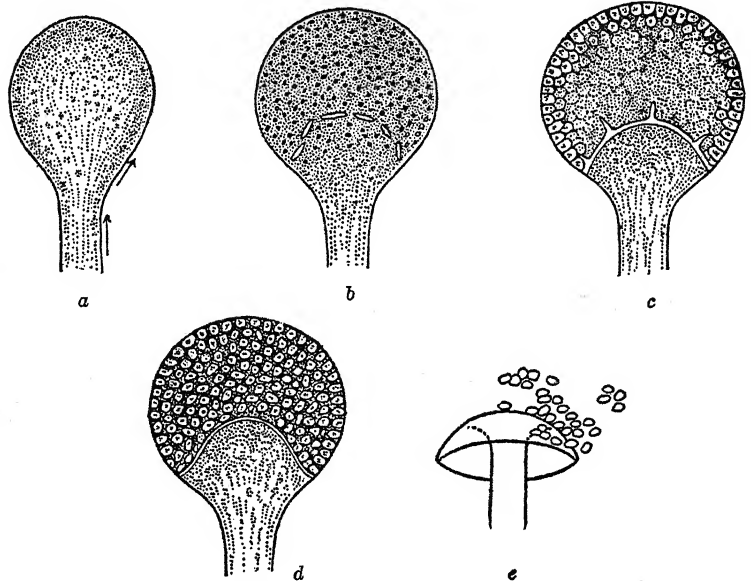


FIG. 8. Illustration to show the development of the spores and columella in *Rhizopus*

a, young sporangium, showing nuclei arranged around the periphery; *b*, formation of vacuoles which later fuse; *c*, columella cleft formed; cleavage of peripheral zone to form spores; *d*, mature sporangium, containing ripe spores; *e*, the columella collapsed; a few freed spores

called zygospores. These result from the conjugation, or union, of two adjacent terminal hyphæ belonging to different plants. Slightly club-shaped branches, similar in appearance and size, come into contact, and a terminal cell called a gamete is cut off by a cross wall from each tip. The double wall now separating the two gametes is dissolved away by an enzyme, and their contents mingle, the nuclei uniting in pairs. The

wall becomes thickened, and rough, and results in a dense, black, irregular-shaped cylinder, to which the name of zygospore is given. When conditions which are suitable for growth arise again, the zygospores germinate to form new plants.

We have noted that the conjugating cells are identical in appearance. In function they are fundamentally different, as hyphæ from distinct races must meet to get zygospore formation. The races have been designated + and - rather than female and male, and we have sexuality rather than a true sex phenomenon represented. Closely related molds belonging to the same class show distinct differences between the male and female cells, conjugation resulting in the formation of oöspores.

Penicillium. We may next take up an example of the Ascomycetes. There are species of these that have a very wide dissemination in nature and are frequently encountered in the bacteriological laboratory. Perhaps the most common one is *Penicillium glaucum (expansum)*, one of the green fruit molds. If we place a piece of apple in a moist, warm chamber, the chances are

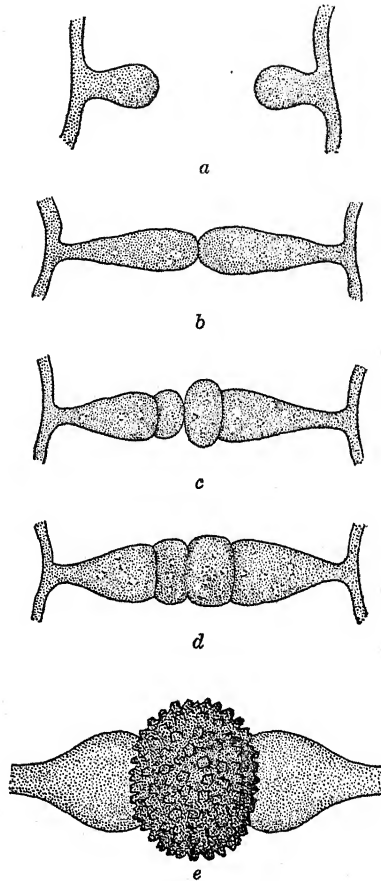


FIG. 9. Zygospore formation in *Rhizopus*

a, b, the approach of two hyphæ; *c, d*, the cross wall is formed, cutting off a cell, or gamete, in each; *e*, the gametes have fused to form the thick-walled zygospore

that within four or five days a rather dense white growth of very fine mycelium will appear on the surface. This will gradually become a delicate light-bluish green and later a darker gray-green color. This is probably a growth of the mold mentioned above. The delicate white threads penetrate

into the fruit tissue for food, while the visible growth is partly vegetative but chiefly composed of fertile hyphæ, conidiophores, which will bear the seeds or asexual spores, the conidia. The growth is not spreading and cottony as in *Rhizopus*, but with due care and patience some of it may be lifted from the surface and mounted for microscopic study.

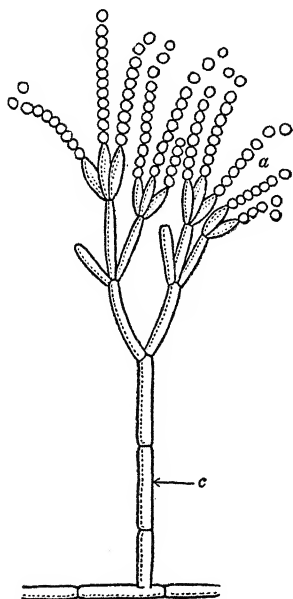


FIG. 10. *Penicillium*, showing how conidia are borne

a, conidiospores; c, conidiophore

The hyphæ are very delicate compared with those of *Rhizopus*. They are colorless, the contents slightly granular, and are divided by septa into cylinders. Many short and long beadlike chains are seen, some free, others attached to special branching hyphæ. These are the conidia, which are slightly elliptical in shape, with a smooth, relatively thick wall.

The conidiophores are seen to branch and rebranch near the ends, so that in a mass they give a broom-like appearance, from which the name *Penicillium* (Lat., "little brush") is derived. The terminal cells of the branchlets are the *sterigmata* (singular, *sterigma*), and the successive conidia are abstricted from them, the oldest conidia therefore being the terminal ones. Under favorable conditions, as when growing naturally on fruit, masses of the conidiophores may unite into a sort of stalk with thousands of spores at the tip, the mass being visible to the unaided eye. Such masses are termed *coremia*.

Penicillium includes hundreds of species, which are among the commonest of the molds. While being classified with the Ascomycetes, there are only four or five of them known to reproduce by the formation of ascospores. To illustrate this important life cycle, which is characteristic of an enormous group of the fungi, we may briefly consider the essential points in one of the mildews, *Pyronema*.

The male and female cells, known as antheridia and oögonia respectively, arise on terminal cells side by side. An outgrowth from the oögonium meets the antheridium, the cell wall is dissolved, and the antheridial nucleus passes over to fertilize the egg cell. The single nucleus arising from the fusion of the male and female nuclei divides and gives rise to eight ascospores within a sac, the ascus. There is true sexuality here, and the nuclear changes examined in more detail suggest strongly an alternation of generations, such as we find in liverworts, mosses, ferns, and all higher plants. The ascospores germinate and grow into a typical mold plant bearing the asexual spores.

Practical importance of the molds. The spores of molds are very widely dispersed in nature, owing (1) to the prodigious numbers in which they are produced by the mold plant, (2) to their buoyancy, permitting them to be wafted through the air to considerable distances, and (3) to their viability, or ability to survive for a long time without germinating, even though dried, frozen, or otherwise exposed to unfavorable conditions. Molds are readily destroyed by boiling temperatures, and so are killed by ordinary cooking, which is not true of all bacteria.

The conditions that favor the growth of molds ordinarily met with in the laboratory or home are moisture, abundant carbohydrate food, a moderate temperature, and absence of bright light. Molds will grow in a strongly acid medium, and in sugar concentrations that ordinarily inhibit the growth of bacteria. Yeasts are like the molds in these respects.

Molds are both injurious and beneficial to man. The majority of plant diseases are caused by fungi conveniently grouped

with the molds. Mildews, rusts, and smuts that damage food-bearing plants often cause extensive economic losses. Their control by the use of chemical sprays or powders, or the propagation of resistant races of plants, is an important branch of scientific agriculture.

Certain molds also cause diseases in animals. A common lung disease of birds (Pneumomycosis) is caused by an *Aspergillus*. There are several infections in man caused by molds. Thrush is an infection of the mucous membranes, chiefly in infants, caused by a fungus (*Oridium albicans*). Several skin diseases (dermatomycoses), of which ringworm and athlete's foot are the most common, are also mold infections.

For foods to become moldy is an all too common experience. Molds are the principal agents of spoilage in natural fruits, as apples, oranges, or muskmelons, and of vegetables, such as tomatoes or potatoes. Starchy cooked foods, as bread or cake, readily become moldy in a damp atmosphere, and molds are prone to occur in jellies and jams or in improperly sealed jars of tomatoes. Molds do not grow without atmospheric oxygen, so their initial growth usually occurs upon the surface.

Linen clothes, tents, or other fabrics, folded while damp and left for a few days in warm weather, become stained and damaged by molds. Even such durable articles as leather shoes or book bindings may become moldy if the air is damp and warm.

Molds are beneficial in that they play a part in soil fertility. They break down woody fiber especially, but are present in rich soil, and, though not fully understood, are unquestionably of great importance in the cycle of elements in organic nature.

Molds play an essential part in the preparation of certain foods, notably cheeses, where they are responsible in large degree for the desired flavors and consistency. The *Penicillium roquefortii* is a well-known example.

This survey is by no means complete, but we shall return to a more detailed consideration of mold activities in other sections of the book.

KEY TO THE MOST COMMON MOLDS

Order Phycomycetes

A. Spores (usually) borne in a sporangium, or spore case. Vegetative hyphæ without cross walls

Family I. Mucoraceæ

a. Sporangiophores arise in clusters or whorls, joined by stolons, or runners *Rhizopus*

b. Sporangiophores arise singly and may or may not be branched. Stolons absent *Mucor*

Order Ascomycetes

B. Spores not borne in sporangia, but free; called conidia. Vegetative hyphæ have cross walls

1. Conidiophores separate; not united into masses

Family II. Mucedinaceæ. Hyphæ not colored; conidia white or colored

a. Conidia one-celled. Conidia formed by segmentation of hyphæ; no distinct conidiophores *Oöspora*

Conidia on distinct branches; often pink or salmon-colored *Monilia*

Distinct conidiophores; conidia in chains. Conidiophores swollen at apex *Aspergillus*

Conidiophores branched. *Penicillium*

b. Conidia two-celled *Trichothecium*

Family III. Dematiaceæ. Either hyphæ or conidia, or both, dark-colored. Conidia one-celled, two-celled, or many-celled

a. Conidia one-celled in chains, easily dispersed . . . *Torula*

b. Conidia many-celled in chains, formed by longitudinal and cross walls *Alternaria*

2. Conidiophores united into masses

Family IV. Stilbaceæ. Conidiophores fused into bundles

CHAPTER III

THE YEASTS

Yeasts or Saccharomycetes (Blastomycetes). Yeasts are among the oldest cultivated plants, references being found in the earliest Biblical and other literature to their use in the making of leavened (yeast-raised) bread, and in the preparation of fermented beverages. They have played a significant rôle in the history of civilization, being at the root of the problem of intemperance with all its social, economic, and moral problems, as well as being indispensable in the making of the "staff of life" — bread. The bread, or brewers', yeast is found only under cultivation.

Yeasts are classified by botanists with the Ascomycetes, indicating that the majority of them, if not all, form sac spores. They differ from the molds in that the true yeasts do not grow in filaments and that their common, asexual method of reproduction is by a process known as budding. We may characterize yeasts, then, as one-celled, colorless plants which reproduce asexually by budding and may form ascospores. There are yeasts which reproduce by fission — the Schizosaccharomycetes. We usually associate the growth of yeasts with alcoholic fermentation, and this used to be thought a criterion for classification. While it is true that the common yeasts produce this kind of chemical change, it is not an essential taxonomic character.

We may take as our type the bread, or brewers', yeast, *Saccharomyces cerevisiæ*. The young cells are round to oval in shape. The internal structure reveals a rather dense cytoplasm, usually with a large single vacuole, but sometimes with several small vacuoles. Each cell contains a nucleus, which may be seen in specially stained preparations. Within the vacuoles are tiny, bright particles, the metachromatic granules.

Actively growing yeast cultures show many of the cells to have small or large protuberances, or knobs. Often there may be two or several such knobs to a cell. These are the buds. In budding, the mother cell first shows a slight bulge on the surface. This gradually swells, becoming constricted at the juncture with the mother cell. The cytoplasm gradually streams into this new bud, and at a certain stage the nucleus elongates, appearing like a dumb-bell, one knob entering the new cell by the narrowing connecting neck. The elongated

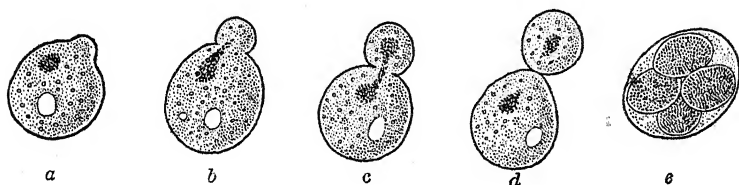


FIG. 11. Yeast cells

a, b, c, d, successive stages in reproduction by budding; e, reproduction by ascospores

nucleus parts company in the middle, one part remaining in the mother cell, the other in the new cell. The constriction now closes and the new cell is an independent being.

In young yeast cultures, grown under special oxygen and temperature relations that suppress budding, ascospores are formed. In *S. cerevisiæ* there is no conjugation of cells preceding spore formation, cytological changes occurring which result in the division of the nucleus and the formation within the modified cell, the ascus, of four resting ascospores. In other yeasts, notably the Schizosaccharomycetes, a sexual process occurs. Two adjacent cells connect by slender protuberances into which the nuclei migrate and fuse. This is followed by a swelling of the connecting neck, and the nucleus divides within the forming sac, usually two or three times, to form four or eight ascospores.

The *Torulaceæ*, sometimes called "wild" yeasts, do not produce spores, but frequently form filamentous-like chains of cells, and form a scum on the surface of pickling fluid. They do not cause alcoholic fermentation. The *Torulæ* are

often pigmented, and red or pink forms may be found as contaminants in bacterial cultures in the laboratory.

Rather recently yeasts which produce exogenous spores have been described. A very rudimentary mycelium may be formed from which arise basidiospores or conidia such as have been described in the last chapter.

Occurrence and character. Yeasts are very widely distributed in nature — in the soil, floating in the air, and especially on the surface of ripening fruits, as grapes or apples. It is inevitable that the expressed juices of fruits, if left at a suitable temperature, will undergo alcoholic fermentation due to yeasts. Yeasts that occur naturally in this way are sometimes called "wild" yeasts, to distinguish them from the more familiar cultivated yeasts that are used in home and commercial fermentations, as bakers' yeast and brewers' yeast; the latter are strains of *S. cerevisiæ*.

The wine yeast, *Saccharomyces ellipsoideus*, is typically a wild plant. It is smaller, more elongated, and the cells tend to cling together more in masses. There are many varieties of *S. ellipsoideus* occurring on natural fruits in particular regions, and the special bouquet, or flavor, of wines, such as Burgundy or Tokay, is due largely to the variety of yeast which causes the fermentation.

The yeasts are surprisingly resistant to external conditions such as drying, freezing, and sunlight. They have been found alive after eight and ten years in cane-sugar solutions, and in beer-wort solutions living cells have been found after thirty years. They are readily destroyed by heat (at 55°–60° C.), the ascospores being somewhat more resistant. Yeasts not only survive but thrive in relatively high concentrations of sugar and in alcohol which may be of their own creation. They grow through a wide range of temperatures.

Yeasts require free or condensed water for their growth. They may grow either in the presence or the absence of atmospheric oxygen.

Practical considerations. The making of bread is familiar to everyone. The yeast plant plays an essential part in bread-

making, as it largely determines the texture, and to some extent the flavor, of the loaf. The fermentation in dough is characteristic of the fermentation caused by yeasts of economic importance, namely, the splitting of sugar (dextrose) into alcohol and carbon dioxide, the latter by-product collecting in innumerable bubbles to make the dough rise. Commercial bakeries represent enormous investments today, and the manufacture of yeast cakes is an industry of some magnitude. The manufacture of beer, ale, wine, and other alcoholic beverages, and the commercial production of ethyl alcohol depend almost solely upon the yeast plant.

Besides these desirable changes the "wild" yeasts may cause undesirable fermentations in beverages or in foods. Some of the "diseases," or abnormal fermentation of wines and beers, studied originally by Pasteur, are caused by yeasts (*Saccharomyces pasteurianus*), while jellies and jams may be spoiled by the growth of yeasts. Any food containing sugar may be fermented by these organisms, and since yeasts are ubiquitous and are relatively resistant to destructive agencies, they are a constant menace to the householder.

The special food value of yeast due to its content of vitamins B (antineuritic) and G (pellagra-preventive) has received a great deal of attention recently. The yeast plant appears to actually synthesize these essential food accessories rather than to concentrate them from the medium in which they are grown. It is doubtful if there is sufficient vitamin in the yeast used in bread-making to be significant. In dried form, or as yeast cake, however, it serves as a fairly rich source of these vitamins.

Irradiation of yeast with ultra-violet light renders it a concentrated source of vitamin D (antirachitic). This has proved especially valuable in feeding dairy cows, as it increases the vitamin-D potency of the milk. The concentration of this vitamin in milk can be controlled by the amount of yeast fed each cow and by determining from time to time the protective value of the milk against rickets in young rats. The use of vitamin-D milk, fortified either in this manner or by direct

irradiation, is becoming rather common with the better grades of milk, and is a distinct contribution to dietetics.

Yeasts have been described as causing certain diseases in man and animals (Blastomycosis). The cases are rare and are of scientific interest rather than of great practical importance.

The pseudo, or false, yeasts, usually grouped under the name *Torula*, do not form spores and produce little or no alcohol. They are prone to occur in solutions containing high concentrations of salt or in pickling solutions.

CHAPTER IV

THE BACTERIA

The bacteria represent a group of very tiny, colorless, unicellular organisms which are commonly classified with plants. The true, or lower, bacteria (Eubacteria) are quite distinct from other organisms on account of their size, simplicity of structure, mode of reproduction, and endospore formation. The more we learn about them, however, the more they fit into the natural scheme of relationship among lower plants. The higher bacteria, or filamentous forms (Thiobacteria), while capable of existence and reproduction from single cells, also may show slight differentiation of cell structure and function. They exhibit branching and methods of reproduction which link the bacteria with the simplest molds and algæ.

The size of bacteria. The unit of measure for microorganisms is the micromillimeter (micron, μ), which is equal to $\frac{1}{25000}$ inch. Bacteria vary greatly in size, some being so minute as to be barely demonstrable with the highest magnifications (some are ultramicroscopic), others being relatively huge (20–30 micra in length by 3–6 micra in width). The average rod cells (bacilli) perhaps range between 1.5μ and 3μ in length and between 0.5μ and 0.8μ in width.

When we contemplate this matter of the infinitesimal size of bacteria, we can appreciate with less incredulity the fabulous size of the figures with which bacteriologists delight to startle the public. It is literally true that a bacterial population greater than the population of New York City may find ample room in a moderate-sized drop of milk, or that 400,000,000 might be packed into the space of an ordinary grain of sugar.

We must caution the reader to think in terms of three dimensions when contemplating the morphology of microor-

ganisms. Too often the student completes a course in general biology with the impression that amœbæ and paramecia are perfectly flat, having only the dimensions observed in the monocular microscope.

Morphology. Bacteria are of three characteristic shapes. These are (1) rod forms, or truncated cylinders, with the ends rounded or occasionally flattened; (2) curved forms, or bent truncate cylinders, with ends variously rounded; (3) spherical, or globular, forms.

To observe the typical shape of an organism is not a matter of random observation upon any culture at hand, but involves

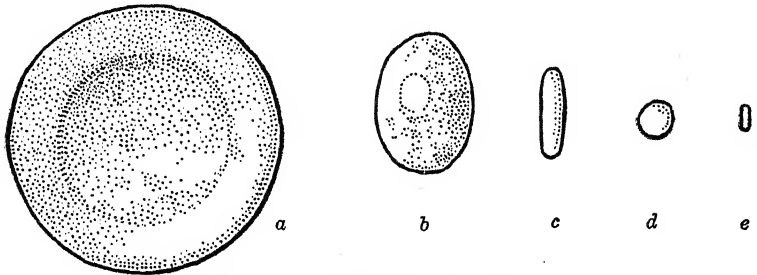
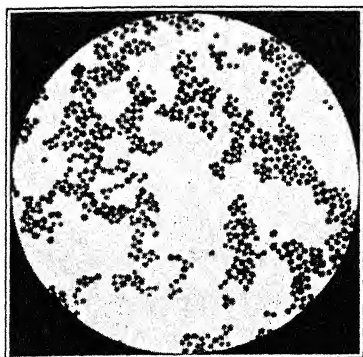


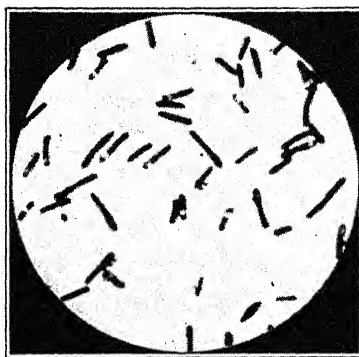
FIG. 12. Relative size of cells

a, human red blood cell; *b*, yeast cell; *c*, typhoid bacillus; *d*, a pyogenic coccus; *e*, so-called influenza bacillus

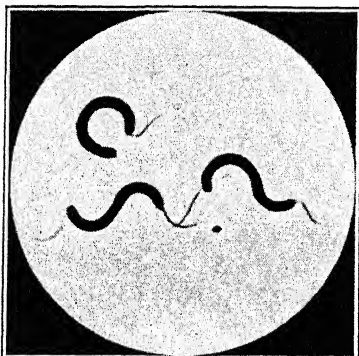
the preliminary cultivation of a known pure culture under certain standard conditions. This is necessary because the age of the culture, its food supply, and other factors may modify morphology and structure. Especially in old cultures, and in those subjected to adverse conditions, monstrosities or strikingly atypical forms may appear. These are called *involution* forms and without doubt represent in most cases degeneration. Such cells are often able to grow and multiply, but are impaired in this regard, as may be shown by transferring them to favorable conditions and observing the slow and meager growth that first takes place. In some instances the appearance of these aberrant forms may be a distinguishing character of positive value, as with the plague bacillus (*Pasteurella pestis*).



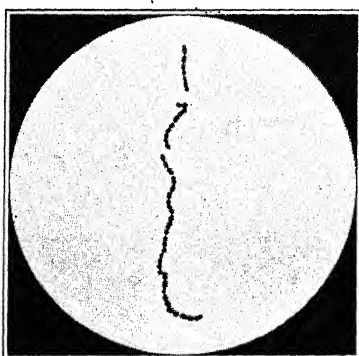
a



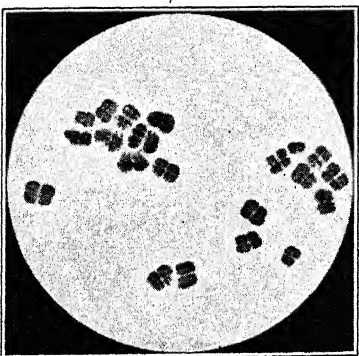
b



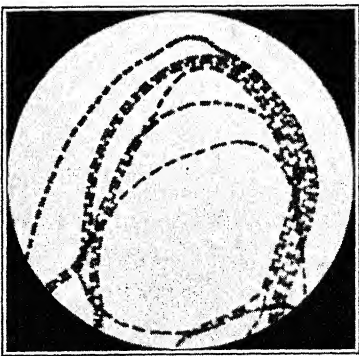
c



d



e



f

FIG. 13. Photographs showing typical morphological types of bacteria
a, staphylococcus; b, *Clostridium botulinum*, showing spores; c, *Spirillum undula*;
d, long-chained streptococcus; e, *Sarcina agilis*; f, *Bacillus anthracis*. (Photographs
by E. Zettnow)

Under uniform conditions of environment bacteria are fairly stable in their morphology. With moderate changes of cultural conditions, or even in the same culture at the same or at different times, organisms which are perfectly healthy, but which deviate radically from the normal, may appear. The presence of bacteriophage also induces certain types of variation or dissociation in bacteria. What shall be said of these? The early view was that the bacteria are extremely

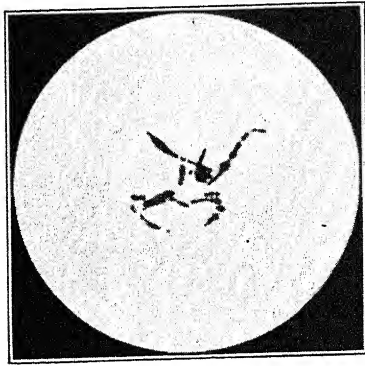


FIG. 14. *Corynebacterium diphtheriae*, showing involution forms

Photograph by E. Zettnow

unstable in all their biological characters, and that one might readily become transformed into another and radically different type. This phenomenon is known as *pleomorphism* (mutability of shape). Koch, perhaps, was the first to insist upon fixity of form. Once a coccus always a coccus, or once a bacillus always a bacillus! We should be cautious in taking too positive a stand on this matter. Certainly the older pleomorphists

were in error; but recent evidence convinces us that rather radical changes can be induced which persist as long as the altered environment persists. Not only may rod forms change or dissociate to spherical forms but they may fragment into minute coccoid bodies that ultimately return to rod forms. Mutations occur in other biologic groups, and although the method of bacteriology is still unsatisfactory to demonstrate these phenomena conclusively, recent work is exceedingly convincing. We shall return to this matter later in the chapter.

The minute structure of bacteria. The minute structure of the bacterial cell is in many respects controversial. The cells are so small that great difficulty is experienced in working out their details. For example, a question so fundamental as the presence or nature of the nucleus is still in debate.

The unstained bacterial cell has slightly greater refraction than water, and appears under the microscope as a colorless or grayish speck showing little or no differentiation. If the cells are motile, the novice is at a loss to know how they "swim," for no organs of motility are visible. Careful and special preparation by staining reveals delicate long compound lashes, the flagella, which propel the motile bacteria. The nature of flagella will be discussed presently.

Bacterial cells are surrounded by *capsules* which have recently been demonstrated to be composed of complex polysaccharides or sugars. These sugars are specific in their chemical properties in very closely related bacteria; even for varieties or types within the same species. The capsule may be so thin as not to be demonstrable, or it may be very thick, embedding the cell in a halo several times the width of the cell itself. The thickness of the capsule varies with the species of bacteria or with the conditions under which the organism is cultivated. In albuminous media, as milk and body fluids, capsule development is encouraged. This gelatinous surrounding is without doubt secreted by the cell. Cultures producing capsules always tend to be viscid, and surprising tenacity is sometimes exhibited. The capsule probably serves as a protective coating, and it also plays a very important part with disease-producing bacteria in determining the reaction they will cause in the animal body. This surprising chemical specificity makes possible the differentiation of closely related bacteria, and it must be taken into consideration in the manufacture of vaccines and anti-sera.

Pneumococcus, *streptococcus*, and other disease-producing bacteria produce capsules, and their virulence, to some extent certainly, depends upon this property. Capsulated organisms are not so readily clumped by serum from immune animals, while destruction of the capsule makes them more vulnerable to such action. Its presence and consistency will determine in part the relation which cells bear to one another after division, whether they adhere in chains or clumps or soon part company.

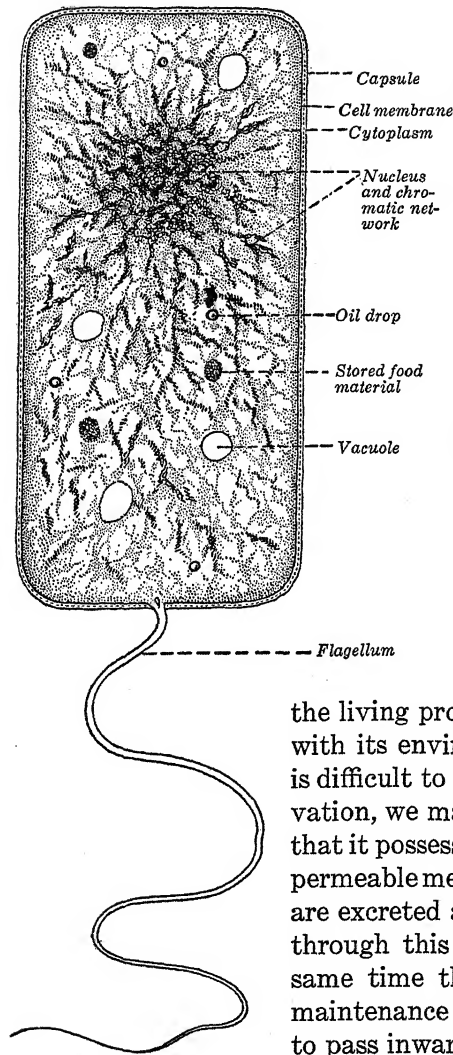


FIG. 15. Diagram, exaggerated to show structure of the bacterial cell

The *cell membrane* has a curious chemical composition resembling chitin, a distinctly animal product, rather than cellulose, the substance of which the typical plant wall is composed. This wall sharply contours the protoplasm and is turgid or stiff, as may be observed when the cell is plasmolyzed, or desiccated, by being subjected to solutes in greater concentration than those within the cell. The cell may retain its original contour while the contents shrink away from the sides.

The cell membrane puts the living protoplasm into relationship with its environment, and although it is difficult to subject it to direct observation, we may conclude from analogy that it possesses the properties of living permeable membranes. Waste products are excreted and enzymes are secreted through this membrane, while at the same time the suitable materials for maintenance and growth are permitted to pass inward through the membrane. As already stated, the cell may be readily plasmolyzed by immersion in salt (1 per cent +) or sugar solutions of sufficient strength, but many cells will endure enormous osmotic tension before they collapse. In solutions of moderate strength

infiltration of the salt occurs and the cell regains its normal appearance. Therefore, although bacteria are quickly affected by changes in concentration, they are also highly adaptable to varying environment. On the contrary, if immersed in distilled water, plasmolysis, or the imbibition of water, occurs, and the cell becomes distended and swollen.

The cell contents from casual observation may appear quite homogeneous. Closer observation or special methods of preparation usually reveal a more or less granular structure of varying size and density; and, in older cells particularly, hyaline spaces or vacuoles occur.

Opinions regarding the *nucleus* in the bacteria are still controversial. Three general views have been expressed: (1) The existence of anything resembling a nucleus has been absolutely denied. (2) The view that there is a typical nucleus showing amitotic division is the opposite extreme. (3) There is the intermediate view of a diffuse nucleus which may at certain stages of the life cycle become more or less condensed. The metachromatic granules first described by Babes (1889) are not the homologues of nuclei, as he supposed, but are composed of volutin, which serves as nonliving reserve material of a transient nature. It tends to accumulate in older cells but disappears during rapid growth. Volutin is a common constituent in the cells of unicellular organisms.

To reach an opinion regarding the nucleus we should consider exactly what we mean by this term. *Nucleus* is essentially a structural term referring to bodies resident in most living cells possessed of certain dynamic potentialities and having a definite chemical composition. Their composition, position, and structure can usually be demonstrated by specific stains.

As has been observed, much loose work has been done on the cytology of the bacteria. Recent careful work has thrown a flood of light on the controversy, and has put to flight the pet theories that bacteria represent more primitive life in their structure than other living forms. Even the smaller bacteria, including cocci, have been demonstrated to possess

the nucleus in some cases. Douglas and Diastaso exclaim: "Es ist kein Zweifel mehr dass alle Bakterien einen Kern besitzen." The chromidial net or diffused granules of chromatin appear in older cells, and possibly may appear only in this form with some bacteria.

The nucleus seems to divide during cell division. It also condenses during spore formation.

The *cytoplasm* assumes an alveolar or network structure in many bacteria, though in some it appears perfectly homogeneous.

Metaplastic granules, starch, oil, and mineral and other matters are found embedded in the cytoplasm. The storage of sulfur and iron as mineral deposits in the cell proper or in the sheath is especially conspicuous in certain of the higher bacteria, and this, in the case of sulphur at least, may explain the origin of the great natural deposits.

The *metachromatic granules* referred to before are now generally considered to play no vital rôle in the cell. Historically they have been interesting, as they were once thought to represent the nucleus. Also it was claimed that they were correlated with virulence in certain types (diphtheria). These curiously deep-staining granules are constantly present in many bacteria, and their presence and position are of importance in the identification of organisms.

Motility. All bacteria, and for that matter other small bodies, as finely divided chalk or fat globules, when suspended in fluids and examined with high-power magnification, exhibit a fascinating dancing or oscillatory movement. This is the result of purely physical forces and bears no relation to vital activities. It is called Brownian or molecular motion.

Besides this movement some bacteria have the power of spontaneously propelling themselves through the suspending fluid by means of motile organs called *flagella* (singular, *flagellum*). These delicate, wavy, threadlike structures are not seen by ordinary observations but require dark-field illumination or special staining for their demonstration. Nonmotile

bacteria do not usually possess these organs. They occur on motile bacilli, spirilla, and the few varieties of motile cocci. Their arrangement on the cell is characteristic for a given variety. There may be a single polar or end flagellum (*monotrichous*) or a tuft of flagella (*lophotrichous*), or, finally, they may be scattered generally over the whole surface in indefinite numbers (*peritrichous*). The length of these lashes is sometimes surprising, being many times the length of the cell to which they are attached. They are apparently very delicate,

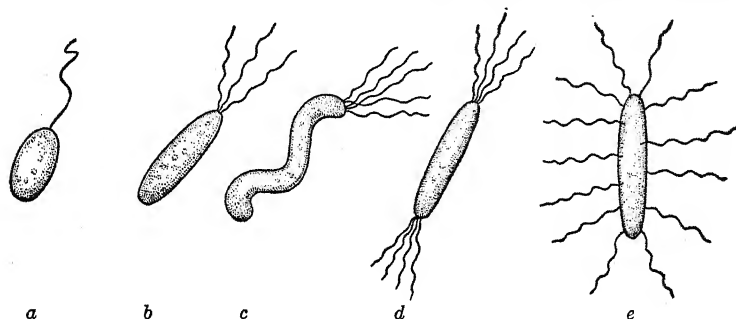


FIG. 16. Bacteria, showing the arrangement of flagella

a, monotrichous; b and c, lophotrichous; d, amphitrichous (rare); e, peritrichous

and are readily broken off from the cell under unfavorable conditions. This probably explains the tangle of these threads sometimes observed apart from, and independent of, cells.

The origin and nature of flagella are somewhat in dispute. They do not seem to be extrusions of the protoplasm, as is the case with many algæ, as may be seen when the bacterial cell is plasmolyzed, for in such cases they may continue to vibrate. One theory is that they are outgrowths from the mucous capsule; but it has been demonstrated that they certainly have some intimate relation with the internal cell, penetrating the membrane through a minute pore, thus establishing a relationship with the internal peripheral zone. A swelling at the base of the flagellum ("basal Korn") has frequently been demonstrated (Führman), but the significance of this has not been settled.

Reproduction. Reproduction among the bacteria takes place by the division of the cell into two equal and identical parts. This is called *fission*. This division, with the rod and spiral forms, always occurs at right angles to the longitudinal axis of the cell. The economy of this is apparent. With the cocci there is little choice as through what diameter the wall will be formed, and we find with certain of the globe forms that fission is haphazard and occurs in any direction. In others it is entirely orderly, and the lines of cleavage are successively parallel to previous lines. Then we may get long series of cells arranged in straight lines or chains; they may be in two planes and at right angles to one another, and will thus give flat masses of cells but one layer thick; they may



FIG. 17. Diagram to show successive stages of fission in bacteria

divide in three planes and at right angles, and thus give clumps of cells in definite blocks or squares. The planes of division, and the tendency of cells to remain in definite groupings, are of importance in determining the relationships of the bacteria, especially the cocci.

Prior to division the rods and spirals elongate to approximately twice the length of the newly divided cell; the cocci do not elongate. The first indication of fission is a slight invagination or constriction of the cell wall. There may be some condensation of granular material in the region of the line of cleavage, and gradually the transverse wall cuts deeper and deeper until it entirely separates the old cell into two new ones. In another place the redistribution of nuclear material has been suggested.

Cells may mature and undergo fission in surprisingly brief periods of time. Under favorable conditions this process may be repeated every thirty minutes or less. It is this prodigious power to multiply that makes it possible for these infinitely small organisms to bring about the rapid and profound

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chemical changes which are observed in connection with their growth. Fischer estimates that a cholera vibrio, which needs only twenty minutes for fission, in a single day "would rejoice in a progeny of sixteen hundred trillions," and that "this mass of bacteria would contain one hundred tons of solid residue."¹ Multiplication in this geometric progression is only theoretically possible and is never maintained for longer than a brief time. The accumulation of waste products, exhaustion of food materials, physical crowding, and other factors very soon restrain this unbridled multiplication. The rapid chemical changes, as the souring of milk in a few hours, are in the same way explicable, not because a few bacteria are very active but because of this surprising ability to grow and to put millions upon millions of cells to work.

Spore formation. Besides this simple fission of the vegetative cells many bacteria have another phase or life cycle — spore formation. We speak, therefore, of spore-formers and non-spore-formers. The bacilli, especially those which grow only in the absence of oxygen, are the most common spore-formers; the spirilla form endospores less commonly; and only in rare instances, if at all, do the cocci form endospores. The spores are always borne within the cell and are called *endospores*. Their formation is encouraged in general by unfavorable environmental conditions, and does not appear to be usually an essential phase of the life cycle, for bacteria may be perpetuated indefinitely without the occurrence of endospores (see Fig. 19). On the other hand, endospores have been found to possess different antigenic or chemical properties from the cells from which they were derived. Also cultures are often somewhat rejuvenated by passing through the spore stage, which suggests that it is more than a simple resting stage. Very rarely is more than one spore produced in a cell, although in some instances, as *Bact. bütschlii*, two endospores are formed at either end of the cell. It is apparent, therefore, that this is not a means of reproduction, as but one (or at most two) new individuals come from the parent cell. It is rather a

¹ A. Fischer, *Structure and Functions of Bacteria*.

method or device for tiding the bacteria over unfavorable circumstances.

Spore formation is foreshadowed by a condensation of the chromatic material in some definite portion of the cell. At the same time the cytoplasm appears more vacuolated. The condensed parts are at first readily stained, but gradually become more resistant to dyes of all sorts and are surrounded

by a very impermeable membrane.

For a given variety the position of the spore within the cell is constant. We speak of it as equatorial when it is both centrally and medially located; as polar when it is at the end or pole of a cell; and as intermediate when it is otherwise located. (If a polar spore shows a swelling of the cell, it is spoken of as capitately; if a centrally located spore bulges

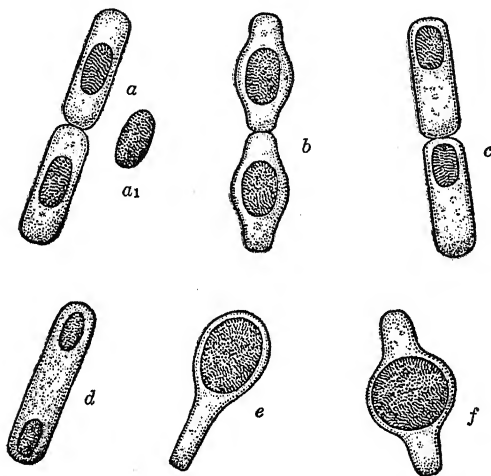


FIG. 18. Diagram to show the position of spores in the bacterial cell

a, equatorial; *a*₁, spore free from the cell; *b* and *f*, clostridial; *c*, polar; *d*, two polar spores (rare); *e*, capitately

the cell, it is called clostridial.) The surrounding cell may persist for some time or may gradually disintegrate, leaving the spore naked.

When the spores are mature they are capable of germinating. As spores are usually produced only under unfavorable conditions, this germination is usually postponed, and may be safely postponed for months or years when the cells are dried. The true endospores of bacteria are highly resistant to unfavorable physical and chemical factors and greatly complicate the processes of sterilization, not only in the laboratory and hospital

but in the home. They may withstand boiling temperatures (no other known living matter can) and also concentrations of chemicals that prove readily destructive to the vegetative cells. Given suitable conditions, they first show a slow swelling and become less refractory and more permeable. Finally the spore wall is ruptured and the new, delicate cell emerges, protruding itself gradually till it frees itself from the old

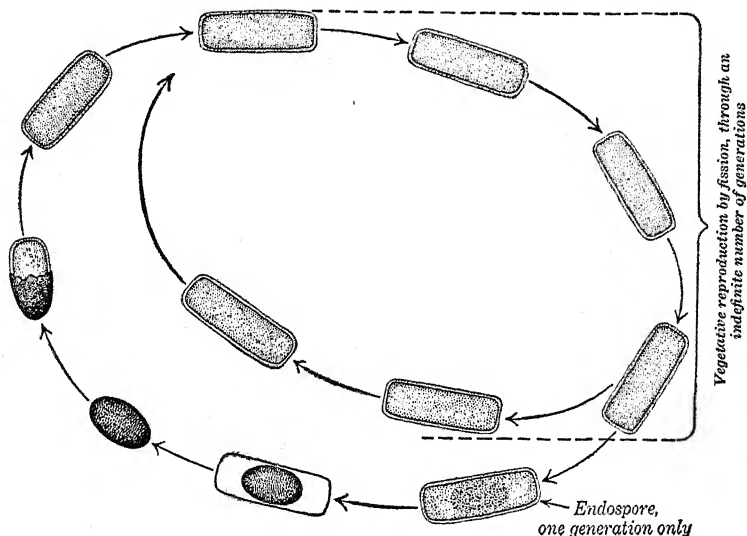


FIG. 19. Plan to show the life cycles of spore-bearing bacteria

sac, leaving it behind as a worthless, ragged remnant. The new cell now starts the vegetative cycle of life anew and for an indefinite period.

It is sometimes stated that a simpler method of spore germination is exhibited by some bacteria, where, without rupturing the old containing sac, the spore itself grows and undergoes fission. If such spores occur, they would be called arthrospores. Their formation is still open to serious doubt.

Life cycles of bacteria. We have already mentioned the variability and instability of the morphology of bacteria. Recent work lends support to the theory that such variability is not always haphazard and meaningless, but that it may,

indeed, be related to rather complicated life cycles of bacteria. The nodule bacteria (*Rhiz. leguminosorum*), found associated with the roots of clover and related plants, are known to change from motile, solid-staining rods to banded, nonmotile rods, which in turn break up into coccus forms, and finally return to the original motile rods. The tubercle bacillus (*Myco. tuberculosis*), streptococci, and other bacteria have been observed by reliable workers to fragment into gonidia, or other minute viable forms, and after suitable cultivation to return to the original recognizable morphological form. Such gonidia, or fragments, are filtrable, that is, they pass through filters which ordinarily retain bacteria. Filtration, however, as a criterion of size or nature of organisms is unreliable because so many factors influence filtrability, such as the electrical charge carried by the filter as well as that carried by the particles to be filtered, the motility and flexibility of the organism, and the pressure or vacuum. Gonidia formation or the formation of other filtrable forms is encouraged by the addition to culture media of lithium chloride, traces of phenol, or other substances, all of which may have an injurious effect upon the cultures. They are also produced by the action of bacteriophage. These discoveries are of great importance, not only from a scientific standpoint, but for practical considerations as well. They may throw light on the nature of the viruses, which, as such, have never been cultivated apart from living cells; and they open up the whole question of the cause of certain diseases, where morphologically typical bacteria are frequently found but where the infecting agent proves to be filtrable.

A still further cycle has been described for a large number, perhaps for all bacteria, where there are not only "filtrable gonidia" but also a stage called the "symplastic" stage, in which the organized cells become disintegrated and amorphous and flow, or "melt," together. From this mass there differentiate the "regenerative units," which grow and become normal cells. The "gonidia" may either be liberated upon dissolution of the cell or they may be budded from the cell, and

they may enter the symplasm or form exospores which germinate into normal cells. It is further claimed that spore-free bacteria may be transformed into spore-forming bacteria, according to "conditions acting upon the symplasm and regenerative bodies."¹

Studies in the heredity of bacteria, by Mellon² and others, confirm the nucleus as an essential structure in bacteria, and also claim a conjugation process resulting in zygosporo formation similar to that explained for yeasts. What bacteriologists have called involution forms, representing degenerative changes, take on a new significance as representing a phase in the life cycle. While we do not yet accept conjugation or sexual reproduction of bacteria as proved, we certainly cannot deny its possibility.

From this hasty survey of the bacteria it will be seen that while the number of shapes which the bacteria exhibit are limited and very simple, and while the demonstrable structures are few, the cells are nevertheless very varied and very complex. It is owing to their minuteness and the consequent inherent difficulties involved in the study of bacterial cells that we have to surmise so much. The routine microscopic examination reveals little more than the gross external dimensions of bacteria, and bacteriologists for the most part have been too busy studying the activities of bacteria to devote themselves to the painstaking problem of minute structure. As this problem is approached more and more in the searching scientific spirit of the systematic botanist and cytologist we must be prepared to modify what have become our traditional views.

Bacteriophage. The most controversial discovery, and one of the most important, in bacteriology in the present century is that of the agent, called bacteriophage, which causes the destruction of bacteria, but which in the process regenerates itself.

¹ Lohnis and Smith, in *Journal of Agricultural Research*, July 31, 1916.

² R. R. Mellon, in *Journal of Bacteriology*, Vol. X (1925), No. 6; Vol. XI (1926), No. 5.

When bacteriophage is added to cultures, it causes lysis, or dissolving of most of the cells present. Young broth cultures that are turbid, owing to the presence of large numbers of bacteria, become clear within a few hours. If planted with bacteria on the surface of solid media, plaques or clear lysed spots appear in, or on the edges of, the colony, causing it to appear "moth-eaten." If bacterial cells being acted upon by bacteriophage are observed under the microscope, they are seen to become swollen, owing to the imbibition of water; the contents appear granular, and finally the cell wall ruptures. Bacteriophage acts chiefly upon young cells, and there are always some cells present in the culture that resist lysis. It can be transmitted from culture to culture indefinitely, as small an amount as one ten-millionth of a cubic centimeter providing sufficient of the agent for such a transfer.

Bacteriophagy is described by D'Herelle as the "*dissolution* of bacteria through the operation of a principle which I have termed *bacteriophage*." The importance of this phenomenon, though observed and described first by Twort, came into its present prominence as the result of the work and publications of D'Herelle in 1922. While the nature of the "principle" involved is still in dispute, the *facts* of bacteriophagy are no longer questioned, and it has become one of the outstanding current problems of investigation.

Bacteriophage has been isolated from a wide variety of sources, including soil, river water, and the body fluids and excretions. It is not only present in the intestinal contents of normal animals and man, but is especially concentrated and active in those convalescent from bacterial infections. This has led to the concept that bacteriophagy may play a part in recovery and immunity from infections. This theory has received support from D'Herelle and others, who have shown that by feeding or inoculating "virulent cultures" of bacteriophage in such diseases as fowl typhoid (caused by *B. gallinarum*), barbone of the Indian buffalo (caused by a *Pasteurella*), and even typhoid, cholera, and staphylococcus infections in human beings, convalescence is hastened, or

resistance to infection is increased. In some instances it is claimed that these effects have been remarkable. However, other investigations have failed to show any benefit from the administration of bacteriophages. Some believe the favorable results to be due to the antigenic properties of bacteriophage itself, or to the disintegration products of bacteria upon which it has acted, rather than to the destructive action of bacteriophage toward the specific infectious bacteria.

The higher bacteria. In addition to the "true bacteria" already discussed in some detail there are five orders of so-called "higher bacteria," to which must be given brief consideration.

Next in importance to the Eubacteriales is the order Actinomycetales, made up of simple organisms, sometimes filamentous, which branch. They do not form endospores, nor do they possess flagella. Some of them form definite branched mycelium and produce growths that strongly suggest molds. Numerous members of this order are found in the soil, giving rise to slow-growing colonies that are often strikingly colored. Of chief interest and importance are the forms that are parasitic on plants and animals. One family, the Mycobacteriaceæ, are very closely related to the true bacteria, showing branching only occasionally. They are all parasitic, and include such important forms as the diphtheria and tuberculosis organisms.

The iron bacteria (*Chlamydobacteriales*) show for the first time in the bacteria a cell differentiation. The base cells in some forms are relatively small and serve to attach the filaments, while the terminal cells may become differentiated to form spores or conidia. Many are surrounded by a sheath in which iron is deposited as an oxide.

The sulphur bacteria (*Thiobacteriales*) occur in water or sewage which contains sulphur compounds, as hydrogen sulphide, and have the remarkable property of reducing and storing the sulphur in pure form within the cell. It serves as a reserve food and is used up when the bacteria are deprived of sulphur compounds in the surrounding medium. They play a rôle in sewage purification. One of the typical forms, *Beggiatoa alba*, shows a swaying and spiral motion as it seems

to creep along, which resembles the unusual motion exhibited by certain of the simple blue-green algæ.

The swarming bacteria (*Myxobacterales*) secrete a gelatinous substance and form a resting stage called a cyst. They are of great interest but contain no members of practical importance.

Finally there is a group of organisms (*Spirochætales*) usually classified as bacteria, but which have many of the characteristics of protozoa. They are long or short flexuous spirals which are actively motile owing to sinuous and rotating movements of the cell itself, and probably never possess flagella. They reproduce by transverse division, and do not form endospores. They include some very important parasitic forms in lower animals and man, including the causative agent of syphilis, *Treponema pallida*.

CHAPTER V

THE CLASSIFICATION OF THE BACTERIA

The classification of bacteria has been, and still is to some extent, in a confused and unsatisfactory state. This is due to a number of causes, but chiefly to the attempt to base relationships solely upon morphology, which is the traditional and logical basis for the larger plant and animal forms. Physiological characters are quite as fundamental and stable, and with the inherent difficulties involved in distinguishing structural differences in organisms of microscopic size it has become necessary and desirable to use a dual standard — the visible structural characters for distinguishing broad general groups, and functional characters for the closer subdivisions. In 1917 the Society of American Bacteriologists attacked the problem through a committee of experts, and this committee published, and has frequently revised, a Manual for the identification of bacteria. Every laboratory should have this Manual available for reference.¹ We shall follow their recommended classification and nomenclature throughout this text. The committee has been guided by the principles laid down by botanical nomenclature, which insists upon the binomial, or two-named, designation of each species: the genus name, which is equivalent to our family name, comes first and begins with a capital letter; the species name, which is equivalent to our Christian name, stands next and begins with a small letter.

The names of orders bear the suffix *ales* (*Eubacteri-ales*); families, the suffix *aceæ* (*Bacill-aceæ*); tribes, the suffix *eæ* (*Nitrobacteri-eæ*). In assigning names priority is to be given, as far as possible, to the person who first discovered or described the organism.

¹ D. H. Bergey, Manual of Determinative Bacteriology, Fourth Edition, 1934.

With these and other principles clearly in mind it has been possible to give a rational classification and nomenclature to the bacteria, doing away with the awkward trinomial and quadrinomial names hitherto used, and increasing the number of genera so as to reduce the confusion caused by the enormous number of species formerly included in one genus.

There follows a selected and abstracted classification taken from the committee report, which gives some idea of the position of a few of the most common or most important bacteria.

CLASSIFICATION OF THE CHIEF TYPES OF SCHIZOMYCETES (FISSION PLANTS)¹

Typically unicellular plants; cells usually small and primitive; many shapes; multiply by fission; some species form endospores; never producing chlorophyll; may be motile by means of flagella. (Pages refer to Bergey's Manual)

	PAGE
Order I. <i>Eubacteriales</i> (true bacteria): Simple undifferentiated spheres, rods, or curved rods; no true branching	29
Family I. <i>Nitrobacteriaceæ</i> : Obligate aërobes; direct metabolism of C, H, N, S, or compounds; no endospores; habitat, soil or water	30
Tribe I. <i>Nitrobacteriæ</i> : Oxidize simple compounds of carbon or nitrogen	31
Includes:	
Genus 4. <i>Nitrosomonas</i> : Rod-shaped; polar flagella; oxidize ammonia to nitrates. Example, <i>Nitrosomonas europæa</i>	34
Genus 7. <i>Acetobacter</i> : Rods; obligate aërobes; oxidize alcohol to acetic acid. Example, <i>Acetobacter aceti</i>	38
Tribe II. <i>Azotobacteriæ</i> : Fix free atmospheric nitrogen	38
Includes:	
Genus 9. <i>Azotobacter</i> : Rods or cocci; obligate aërobes; oxidize carbohydrates and fix (oxidize) nitrogen. Example, <i>Azotobacter chroococcum</i>	43
Genus 10. <i>Rhizobium</i> : Rods; branching forms; obligate aërobes fixing (oxidizing) nitrogen in symbiosis with, or as parasites of, plants. Example, <i>Rhizobium leguminosarum</i>	44
Family II. <i>Coccaceæ</i> : Cells spherical. Division in one, two, or three planes; occur singly, in pairs, chains, tetrads, packets, or masses. Rarely motile; no endospores; metabolism complex; often produce pigment.	47

¹D. H. Bergey, Manual of Determinative Bacteriology, Fourth Edition, 1934.
(Arranged by a committee of the Society of American Bacteriologists.)

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Tribe I. <i>Streptococcæ</i> : Cell division one plane. Mostly parasites; pairs or chains; growth scant; Gram-positive	48
Includes:	
Genus 1. <i>Diplococcus</i> : Parasitic; usually in pairs; encapsulated; active fermenters. Example, <i>Diplococcus pneumoniae</i>	48
Genus 2. <i>Streptococcus</i> : Chiefly parasites; cells in pairs or chains; rarely encapsulated; growth meager. Example, <i>Streptococcus pyogenes</i>	50
Tribe II. <i>Neisseriæ</i> : All parasites; cells in pairs; Gram-negative	68
Includes:	
Genus 4. <i>Neisseria</i> : Characters of the tribe. Example, <i>Neisseria gonorrhæa</i>	69
Tribe III. <i>Micrococccæ</i> : Parasites or saprophytes. Cells single, in packets, or in regular masses; Gram-positive or Gram-negative; many produce yellow or red pigment	72
Includes:	
Genus 5. <i>Staphylococcus</i> : Usually parasites; cells, single, pairs, or irregular masses; grow fairly well in artificial media. Example, <i>Staphylococcus aureus</i>	73
Genus 7. <i>Micrococcus</i> : Facultative parasites or saprophytes; cells in plates or irregular masses; usually Gram-positive. Example, <i>Micrococcus luteus</i>	78
Genus 8. <i>Sarcina</i> : Usually saprophytes; division in three planes to form regular packets. Example, <i>Sarcina lutea</i>	101
Family III. <i>Spirillacæ</i> : Elongated and more or less spiral rods; no endospores; usually motile with polar flagella; typically water or intestinal parasites	109
Genus 1. <i>Vibrio</i> : Short, bent rods; motile; single polar flagellum. Example, <i>Vibrio comma</i> (cause of Asiatic cholera)	110
Genus 2. <i>Spirillum</i> : longer spirals. Example, <i>Spirillum rubrum</i>	120
Family IV. <i>Bacteriacæ</i> : Rod-shaped; without endospores; motile or nonmotile; complex metabolism; usually Gram-negative	122
Tribe I. <i>Chromobacteriæ</i> : Produce pigments; aerobic saprophytes and plant parasites	123
Genus 1. <i>Serratia</i> : Produce red or pink pigments on solid media. Example, <i>Serratia marcescens</i>	123
Genus 2. <i>Flavobacterium</i> : Produce yellow or orange pigment on solid media. Example, <i>Flavobacterium aquatile</i>	135
Genus 3. <i>Chromobacterium</i> : Produce violet pigment on solid media. Example, <i>Chromobacterium violaceum</i>	165
Genus 4. <i>Pseudomonas</i> : Produce water-soluble, diffusible green, blue, or yellowish-green pigments; parasitic and saprophytic. Example, <i>Pseudomonas æruginosa</i>	174
Tribe III. <i>Cellulomonadæ</i> : Do not commonly produce pigment; digest cellulose	193

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Tribe V. <i>Erwinia</i> : Plant pathogens	248
Includes:	
Genus 8. <i>Erwinia</i> : Motile, peritrichous flagella. Example, <i>Erwinia amylovora</i> (causes "blight" in pear and apple trees)	249
Genus 9. <i>Phytomonas</i> : Motile and nonmotile; polar flagella. Example, <i>Phytomonas campestris</i> (causes "black-rot" of cabbage and related plants)	254
Tribe VI. <i>Lactobacillæ</i> : Usually long, slender rods; Gram-positive; nonmotile; no endospores; always produce lactic acid from carbohydrates	300
Includes:	
Genus 10. <i>Lactobacillus</i> : Characters those of tribe. Example, <i>Lactobacillus acidophilus</i> ; habitat, the intestine	300
Tribe IX. <i>Pasteurella</i> : Aërobic; bipolar staining; pathogenic for lower animals and man. Example, <i>Pasteurella pestis</i> (causes plague in man and rodents)	329
Tribe XI. <i>Hemophilæ</i> : Minute, nonmotile, sometimes pleomorphic, Gram-negative rods; parasites growing only in presence of hemoglobin, or body fluids, or plant tissues	337
Includes:	
Genus 15. <i>Hemophilus</i> : Strict animal parasites. Example, <i>Hemophilus influenza</i>	337
Tribe XII. <i>Bacteriæ</i> : Gram-negative rods, chiefly of intestinal tract; grow well on artificial media; many attack carbohydrates to form acid, or acid and gas	342
Includes:	
Genus 8. <i>Escherichia</i> : Form acid and gas in lactose and other sugars. Example, <i>Escherichia coli</i>	342
Genus 19. <i>Aërobacter</i> : Differ from <i>Escherichia</i> in power to form acetylmethylcarbinol. Example, <i>Aërobacter aërogenes</i>	356
Genus 20. <i>Proteus</i> : Highly pleomorphic; ferment dextrose and sucrose, but not lactose. Example, <i>Proteus vulgaris</i>	361
Genus 21. <i>Salmonella</i> : Attack many sugars, but not lactose, sucrose, or salicin. Cause of various intestinal infections. Example, <i>Salmonella paratyphi</i>	368
Genus 22. <i>Eberthella</i> : Attack various sugars to form acid but no gas; generally intestinal parasites of man. Example, <i>Eberthella typhosa</i>	381
Genus 23. <i>Shigella</i> : Nonmotile; form acid, no gas; intestinal parasites. Example, <i>Shigella dysenteriae</i>	388
Genus 24. <i>Brucella</i> : Rods and coccoid cells; nonmotile; form neither acid nor gas from sugars; parasitic in animals. Example, <i>Brucella abortus</i>	397
Genus 25. <i>Alcaligenes</i> : Nonfermenters; intestinal forms; nonpathogenic. Example, <i>Alcaligenes faecalis</i>	399
Family V. <i>Bacillaceæ</i> : Rods producing endospores; usually Gram-positive; decompose protein	414
Genus 1. <i>Bacillus</i> : Aërobic; usually saprophytic. Example, <i>Bacillus subtilis</i>	414

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	PAGE
Genus 2. <i>Clostridium</i> : Anaërobic; often parasitic. Example, <i>Clostridium botulinum</i>	466
Order II. <i>Actinomycetales</i> : Elongated, frequently filamentous rods; often branched and with clubbed ends; endospores not produced, but conidia developed in some genera; non-motile; usually aërobic	495
Family I. <i>Actinomycetaceæ</i> : Filamentous forms; sometimes pathogenic. Example, <i>Actinomyces bovis</i>	495
Family II. <i>Mycobacteriaceæ</i> : Irregularly staining; pleomorphic rods; rarely filamentous; occasionally branching; saprophytes and parasites	534
Genus 1. <i>Mycobacterium</i> : Slender rods; acid-fast. Example, <i>Mycobacterium tuberculosis</i>	534
Genus 2. <i>Corynebacterium</i> : Not acid-fast; uneven staining; Gram-positive; slender rods; often curved, with tendency to club. Example, <i>Corynebacterium diphtheriæ</i>	545
Order III. <i>Chlamydobacterales</i> : Filamentous water forms; often showing false branching; sheath often impregnated with iron	563
Order IV. <i>Thiobacterales</i> : Contain either sulphur granules or bacteriopurpurin, or both; spores rarely or never formed	567
Order V. <i>Mycobacterales</i> : Cells in colonies; secreting gelatinous mobile material; produce spores and other fruiting bodies	588
Order VI. <i>Spirochætales</i> : Protozoan-like; slender flexuous spirals; cell division longitudinal or transverse	617
Includes:	
Genus 1. <i>Spirochæta</i> : Usually saprophytic; in water; spiral wound around a central axis filament	618
Genus 5. <i>Treponema</i> : Parasitic, frequently pathogenic; with undulating or rigid spirilliform body. Example, <i>Treponema pallidum</i> (cause of syphilis)	627
Genus 6. <i>Leptospira</i> : Pathogens; hooked at end. Example, <i>Leptospira icteroides</i> (cause of infectious jaundice)	629

CHAPTER VI

LABORATORY METHODS FOR THE STUDY OF BACTERIA

The minuteness of bacteria makes it impracticable to study the life activities of a single cell. We therefore require methods for growing bacteria in large numbers in order to study their biochemistry. The study implies their growth on a suitable *soil* or in a *culture medium*. The growth is called a bacterial culture. Some bacteria are grown with difficulty on artificial laboratory media, as, for example, the organism causing meningitis, *Neisseria intracellularis*, or that causing whooping cough, *Hemophilus pertussis*. On the other hand, the common saprophyte, *Bacillus subtilis*, grows readily.

A culture containing only one kind of organism is known as a *pure culture*; if several are present, it is called a *mixed culture*. Obviously the study and identification of an organism implies first its isolation in pure culture. This is often a difficult matter and requires the most careful bacteriological technique.

After obtaining a pure culture, special characteristics of the organism must be studied, to differentiate it from others. Morphology may be relied upon in a general way to set apart different genera, but the physiology of the organism must be studied to identify the species.

We cannot stress too strongly the need of careful technique. All glassware, instruments, and nutrient substances must be free from any existing form of life before beginning any experiment. This is the basis of all bacteriological work.

We shall deal in this chapter with the various methods used in the laboratory for the complete study of a microörganism. The principles which will be enlarged upon are

1. Sterilization of materials and apparatus used in bacteriological work.

2. Cultivation of the organism.
 - a. Cultivation of aërobes.
 - b. Cultivation of anaërobes.
3. Isolation of the organism in pure culture.
4. Detailed study of the morphology of individual cells under the microscope in
 - a. Unstained preparations.
 - b. Stained preparations.
5. Animal inoculation.
6. Serum reactions.

Sterilization. The student in elementary bacteriology will ordinarily master only the simple laboratory methods. In this chapter we aim to stress the general principles involved in bacteriological technique. All bacteriological methods are dependent upon the preliminary sterilization of the apparatus and media in which the organisms are to be grown. We know that microorganisms are ubiquitous; they are on our hands, on the laboratory tables and apparatus, and in the dust of the air. Every precaution must therefore be taken to prevent any foreign organisms from coming in contact with, or contaminating, the organisms which we desire to study.

We may define sterilization as any process whereby material is freed from living organisms. Sterilization may be effected by either physical or chemical means. The most common of the physical agents are *heat*, *light*, and *filtration*. The early investigators who sought to disprove the theory of spontaneous generation boiled their decoctions in flasks and showed that, if properly sealed, no fermentation resulted. Later, when it was discovered that some bacteria are extraordinarily resistant to heat, other methods of sterilization were devised which enabled one to use higher temperatures. One must be certain in any method that the temperature, and the time of exposure to that temperature, are sufficient to accomplish the sterilization. The method used is dependent upon the nature of the material to be sterilized.

Sterilization by heat may be accomplished by the use of dry or moist heat. The student is first introduced to the use of

dry heat as a means of sterilization when he thrusts the platinum inoculating needle or instrument directly into the flame. This procedure is obviously of limited application. Dry heat, or "baking," is used to sterilize glassware or materials not injured by this method; it is not suitable for the sterilization of organic substances, with the exception of cotton or paper.

It is important to have all glassware free from any chemical which might be injurious to bacterial growth. Thorough

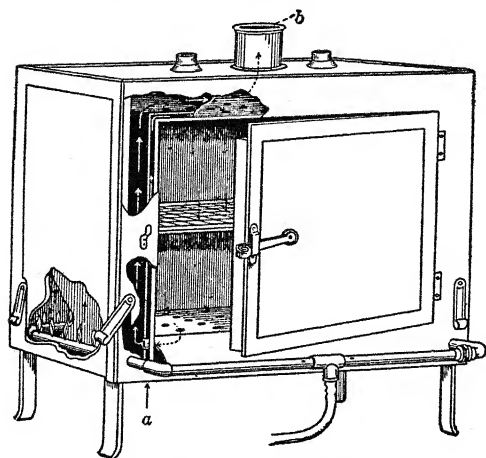


FIG. 20. Hot-air sterilizer

a, inlet, and *b*, outlet for circulating air

cleansing and rinsing of glassware, therefore, is a preliminary step. Glass may be thoroughly cleaned by boiling in 10 per cent nitric acid or by soaking for an hour in a chromic acid cleaning solution. After being thoroughly rinsed and dried, the tubes and flasks are plugged with nonabsorbent cotton. Enough cotton should be used to make a firm plug. Test tubes, when properly plugged, may be picked up, and suspended, by the cotton. The glassware is then ready to be sterilized in a hot-air oven.

One hour exposure at a temperature of 170° – 190° C. is required for the destruction of resistant spores. Pipettes, porcelain apparatus, Petri dishes, and other glassware are sterilized in the hot-air oven. In order to protect pipettes from contamination after removal from the sterilizer, they should be wrapped in paper or placed in copper containers.

Moist heat is more effective in sterilization than dry heat. The temperature obtained by a hot-air oven would decompose organic material such as laboratory media. Fur-

thermore, the concentration of solutes is not great enough to raise the boiling point appreciably; thus the medium would boil over. Sterilization by moist heat may be effected in one of several ways:

1. By the use of streaming steam.

2. By the use of steam under pressure.

3. By intermittent heat at moderate temperatures.

Sterilization by streaming steam is accomplished by an Arnold sterilizer or a similar apparatus which corresponds to an ordinary steamer used in the home. The steam is generated in a pan beneath, circulates up through the compartment, and thus comes in contact with the material to be sterilized. The temperature is that of live steam, 100°C . One exposure at this temperature for fifteen minutes will kill all vegetative cells, but some spores may resist and later germinate. It is customary, therefore, to sterilize on three successive days for fifteen minutes. This process is called *intermittent sterilization*. Its object is to kill, at the second and third exposures, the spores that have germinated between heatings.

We may apply the method of intermittent sterilization in home canning. Various examples of the use of steam or

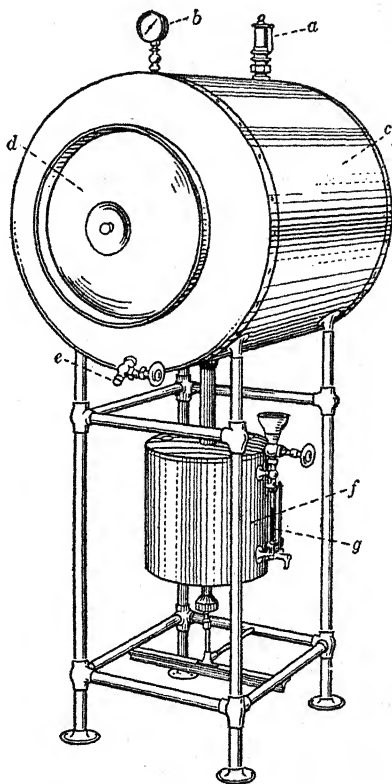


FIG. 21. Horizontal pressure sterilizer, or autoclave

a, safety valve; b, pressure gauge; c, sterilizing chamber; d, rolling door, with ground valve seat; e, outlet for steam; f, steam generator; g, water gauge

scalding water as a means of sterilization in the home and dairy will occur to us.

Steam under pressure, with a temperature varying directly as the pressure (see Fig. 22), accomplishes the sterilization of culture media at a single exposure. The apparatus used for sterilization by steam under pressure is known as the *autoclave*, or digester. It consists of a chamber which may be tightly closed, into which steam under pressure is introduced. The steam is injected at the top of the chamber, displacing

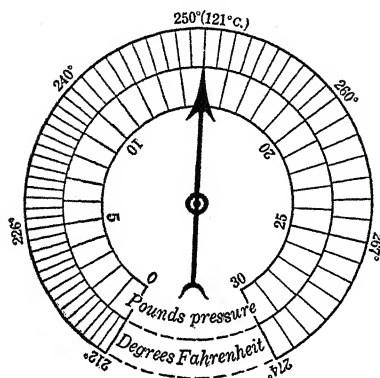


FIG. 22. Pressure gauge on autoclave

the air, which is forced out of the open cock at the lower front end. When steam issues from the cock it is ready to be closed. A temperature of 120° C., and 15 pounds pressure, for fifteen minutes is ordinarily used. The apparatus is provided with a pressure gauge and safety valve which can be set to blow off if the pressure becomes higher. If large flasks of media are used, it is nec-

essary to heat for a longer time to be sure that all the medium has been raised to the required temperature.

Canning factories use autoclaves of special design in the commercial preparation of their canned-food products.

Precautions to be taken in the use of the autoclave are

1. Do not pack material too tightly together.
2. Do not close the cock until live steam spurts out.
3. Allow the pressure to go up gradually.
4. Allow at least fifteen minutes' exposure *after* the pressure has reached the required point.
5. When sterilization is completed, do not open the autoclave until the manometer needle points to zero; otherwise the sudden release of pressure may blow the stoppers, and the media will boil over.

The principle underlying all the devices for *sterilization by filtration* is the same, namely, the straining out of bacteria, leaving a sterile filtrate. We have already spoken of the plugging of tubes with cotton as a method of excluding microorganisms in the outside air. If the plugs have become wet in the sterilization process, they are no longer efficient as filters. Microorganisms can then grow through the plug and contaminate the medium. There are many kinds of filters made of porcelain and infusorial earth, the pores of which are so fine that bacteria are retained while colloids and very minute organisms pass through. The factors which determine the passage of particles through filters are very complex. It is not the size of the pores of the filter alone that determines the retention or passage of particles, but the phenomenon of adsorption, which in turn may be modified by the reaction of the medium. The spaces in the filter may be large enough to permit bacteria to pass and yet will retain them. However, the bacteria may grow through such a filter in a few hours. The filters most commonly used are the Pasteur-Chamberland, the Chamberland, and the Berkefeld. Filters of this character are used in the sterilization of drinking water, in the preparation of therapeutic sera, and in work with filtrable viruses. D'Herelle¹ describes the use of a collodion membrane which retains the bacteriophage "corpuscle."

The destructive action of *light* upon bacteria is taken up more fully in Chapter VIII. Suffice it to say here that, in general, light is destructive to microorganisms. The ultra-violet and blue-violet rays of the spectrum have the most powerful germicidal effect. This has a broad hygienic application; disease-producing organisms cannot long survive outside the body when exposed to the direct rays of the sun.

The Cooper-Hewitt mercury-vapor lamp is used to produce the ultra-violet germicidal light rays and is sometimes used in the sterilization of water and other fluids.

Culture media. To obtain a pure culture requires a suitable medium in which to grow the organism. The bacteriolo-

¹F. D'Herelle, *The Bacteriophage and its Behavior*, pp. 25-34.

gist aims to find what the organism requires in the way of a physical and chemical environment, and then makes the artificial medium to meet these requirements. It is important that the medium have a moist surface. Bacteria are essentially aquatic plants; the cells must be entirely surrounded by water. In a medium slightly acid to phenolphthalein, and at a temperature about 20° C., optimum conditions for the growth of most microorganisms are found. As we shall see, different species of bacteria differ as to their physical and chemical requirements. Many special laboratory media have been devised.¹ It is by the study of the characteristic biochemical changes brought about by bacterial fermentation in these carefully prepared nutrient substances that we are able to identify bacterial species. A satisfactory culture medium must fulfill the following requirements:

1. It must be sterile, and kept in such a way as to prevent contamination from the outside.
2. It must contain all the elements, in an available form, necessary for the growth of the organism.
3. No injurious chemicals shall be present which will inhibit bacterial growth.
4. The medium must be moist.
5. The reaction of the medium must be adjusted to suit the organism.

We may divide culture media, on the basis of consistency, into three types: *liquid*, *liquefiable solid*, and *nonliquefiable solid*. For very accurate work certain *synthetic* media are used which contain exact amounts of chemicals of known composition. Synthetic media are especially important in the study of soil bacteriology.

The most common medium has for its basis a decoction of lean beef, or a prepared extract of beef, to which a small amount of peptone has been added. Koch found that the addition of gelatin to this beef peptone broth gave a clear,

¹ Levine and Schoenlein, *Compilation of Culture Media for Cultivation of Microorganisms*. Also, report of Committee on Standard Methods, American Society of Bacteriology.

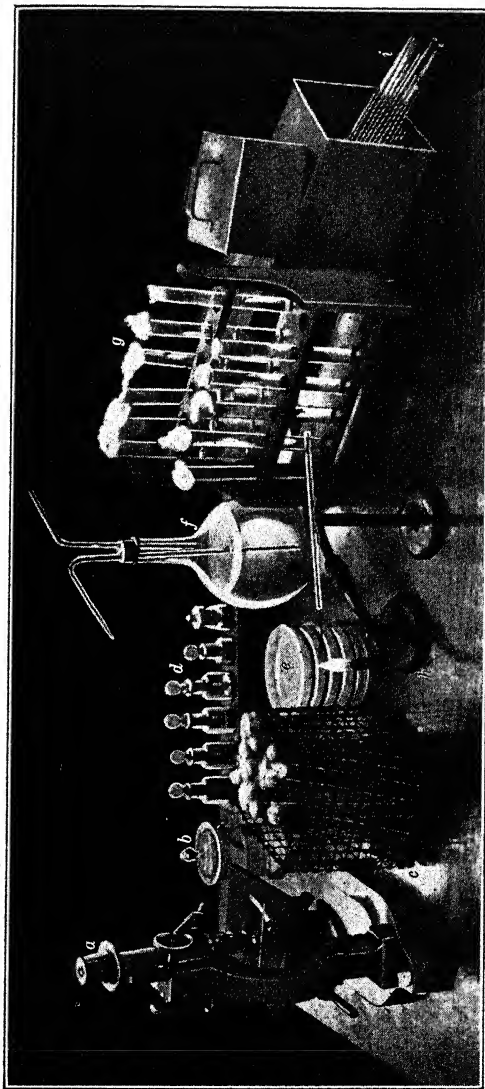


FIG. 23. Equipment essential for cultivating and observing bacteria

a, compound microscope equipped with oil-immersion objective; *b*, slide jar containing glass microscope slides in alcohol (one mounted on the stage of the microscope); *c*, wire basket for holding tubes of culture media during sterilization and storage; *d*, rack holding stain bottles, alcohol, cedar oil, etc.; *e*, Petri dishes; *f*, wash bottle for washing off stains; *g*, test-tube rack holding tubes of media or sterile water; *h*, gas burner for sterilizing inoculating needle (see needle and rest to right); *i*, sterile, graduated pipettes in copper box

solid medium. A liquefiable solid medium is one which is liquid at one temperature and solid at another, as is the case with gelatin and agar. Gelatin does not remain solid at 37 C., the temperature at which most pathogens grow best.

Agar, a nonnitrogenous preparation made from an Oriental seaweed, has the advantage over gelatin in that it remains solid at much higher temperatures. Examples of nonliquefiable solid media are potato and coagulated albumins, such as egg and blood serum.

The introduction of solid media into bacteriology was an important step. There is probably no technique in the study of bacteria which is more important and of wider application than the selection of colonies from the surface of solid media.

The bacteria, finding ample food, begin to multiply; they are, however, fixed in their positions and are isolated from each other. As they multiply they give rise to isolated specks, or *colonies*, on the agar plate. Koch found that if he selected one of these colonies and transferred it to a tube of sterile medium, a pure culture of an organism was obtained.

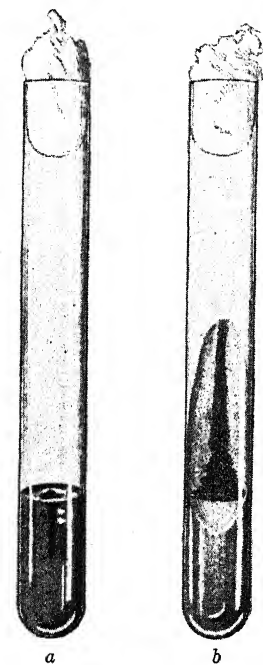


FIG. 24. Typical bacterial growth on agar

a, *Spirillum rubrum*, "stab" culture; b, *Eberthella typhi*, slant culture

The use of solid media is the basis of quantitative bacteriology. The technique of diluting material for bacteriological analysis is taken up later in the chapter.

The characteristic growth of an organism on the surface of a medium gives, moreover, a means of differentiating bacteria. Colonies may be classified as to their size, form, contour, surface elevation, consistency, and internal structure.

For example, a colony may be described as mycelioid, filamentous, or amœboid; by touching it with the needle we may determine whether it is slimy, butyrous, or dry. Colonies on the surface may appear as tiny spots (punctiform); if they are subsurface, their shape may resemble a lens or football set in the medium. We examine the surface elevation of a colony and note whether it is concave or convex. Reference to the list of terms given below will be of assistance in describing colonies.

TERMS USED IN DESCRIBING COLONIES IN PLATE CULTURES

- Amorphous*: without visible differentiation in structure
Arborescent: branched, treelike growth
Brittle: growth dry, friable under the platinum needle
Butyrous: growth of butterlike consistency
Curled: composed of parallel chains in wavy strands, as in anthrax colonies
Entire: with an even margin
Erose: border irregularly toothed
Filamentous: growth composed of long, irregularly placed or interwoven threads
Granular: composed of small granules
Lobate: having the margin deeply undulate, producing lobes
Mycelioid: colonies having the radiately filamentous appearance of mold colonies
Papillate: growth beset with small, nipple-like processes
Plumose: a fleecy or feathery growth
Pulvinate: decidedly convex, in the form of a cushion
Punctiform: very small, but visible to the naked eye; under 1 mm. in diameter
Radiate: showing ray structure
Raised: growth thick, with abrupt or terraced edges
Rhizoid: growth of an irregular branched or rootlike character
Rugose: wrinkled
Umbonate: having a buttonlike, raised center
Undulate: border wavy, with shallow sinuses
Viscid: growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl

TERMS USED IN DESCRIBING STAB OR STROKE CULTURES

Arborescent: branched, treelike growth

Beaded: disjointed or semiconfluent colonies along the line of inoculation

Crateriform: a saucer-shaped liquefaction of the medium

Echinulate: a growth along line of inoculation with toothed or pointed margins

Effuse: growth thin, veil-like, unusually spreading

Filiform: a uniform growth along line of inoculation

Infundibuliform: in form of a funnel or inverted cone

Napiform: liquefaction in form of a turnip

Saccate: liquefaction in form of an elongated sac, tubular, cylindrical

Spreading: growth extending beyond the line of inoculation several millimeters or more

Stratiform: liquefying to the walls of the tube at the top and then proceeding downward in layers

TERMS USED IN DESCRIBING GROWTH IN
LIQUID CULTURES

Flocculent: containing small adherent masses of bacteria of various shapes floating in the culture fluid

Membranous: growth thin, coherent, like a membrane

Pellicle: bacterial growth forming either a continuous or an interrupted sheet over the culture fluid

Ring: growth at the upper margin of a liquid culture, adhering to the glass

Turbid: cloudy, with flocculent particles¹

Carbohydrates, most commonly either dextrose, lactose, maltose, sucrose, mannite, or xylose, may be added to broth or agar to determine their availability as food. Blood and ascitic fluid, which, in composition, approach closely the body tissues, may be used for growing parasitic bacteria. Milk is a valuable natural medium for the growth of bacteria. It is also an important differential medium for bacteria; some form acid from the sugar present and curdle the milk; others attack the pro-

¹ The definitions are taken from "Manual of Methods for Pure Culture Study of Bacteria," published by the Society of American Bacteriologists, Geneva, New York.

tein and digest it. Often we wish to study the change in reaction produced by the organism, and therefore add some indicator to the medium. Litmus milk, or milk containing an indicator such as brom thymol blue, may be used for the purpose; if the organism is an *acid-producer* or causes an increase in the concentration of free hydrogen ions, a distinct color change will result (see Table I, p. 69). Certain dye media are of value in that they are *selective*, allowing the growth of one organism while inhibiting others. A striking example of this selective action is observed in the use of gentian violet dye, which even in very high dilution totally inhibits the growth of the strongly Gram-positive organisms, while not affecting those that are Gram-negative.¹ The presence of other dyes in culture media shows evidence of chemical reactions which are characteristic of certain bacteria, giving us a convenient means of isolation. Thus, eosin and methylene blue added to sugar media readily distinguish between the colonies of the typhoid and colon organisms, the former appearing colorless, while the latter are bluish black.

Adjustment of the reaction of a medium. Microorganisms are sensitive to the presence of excess acid or alkali; it is therefore necessary to adjust the reaction of the medium to suit special needs. Moreover, methods are required to study the change in reaction which an organism may bring about in a medium.

By *reaction* we mean the acidity or alkalinity of a solution. This is determined by the relative concentration of hydrogen ions or hydroxyl ions present. A neutral solution is one in which both radicals are present in equal proportions; an acid solution is one in which there are hydrogen ions in excess; an alkaline solution is one in which there are free hydroxyl ions.

Pure water dissociates to a very slight extent into H ions and OH ions; they are present in equal amounts, and hence the reaction is neutral. The concentration of H ions (C_H) in pure water is 0.0000001, or 10^{-7} grams per liter. The symbol "pH" is used, the pH value of a solution being equal to

¹ For description of Gram stain see page 80.

the log of the reciprocal of the C_H value; that is, $pH = \log \frac{1}{C_H} = -\log C_H$. To express this simply: the pH value is approximately the same as the number of places after the decimal point; the decimal figure itself represents approximately the number of grams of H ions per liter. Thus with an increase in C_H the pH value will decrease; with a decrease in the C_H the pH value will increase. The H ion concentration of pure water is given by the formula $pH = 7.0$; normal NaOH, $pH = 14$; normal HCl, $pH = 0.0$.

We shall consider very briefly the methods by which we may adjust the reaction of a medium. They are

1. Titration by means of phenolphthalein.
2. Colorimetric titration using indicators other than phenolphthalein.
3. Electrometric method by means of the potentiometer.

Phenolphthalein is no longer recommended for titrating media used in bacteriological work. This method is open to many objections on chemical grounds, owing to the presence of *buffers*. Buffers are organic substances, such as peptone, beef infusion, or blood, which interfere with the change in pH value even though the quantity of acid or alkali present has changed considerably. The reaction is dependent upon free H ions, and in the presence of buffers considerable acid or alkali may be added without altering the reaction. Ten cubic centimeters of $N/10$ sodium hydroxide will neutralize ten cubic centimeters of $N/10$ acetic acid and ten cubic centimeters of $N/10$ hydrochloric acid, although the pH of N acetic acid is 2.4 as against $pH = 1.05$ for $N/10$ hydrochloric acid. Tenth-normal hydrochloric acid has approximately 22.5 times the H ion concentration of normal acetic acid.

The colorimetric methods based on H ion concentration are most commonly used and are the most satisfactory for ordinary bacteriological work. Indicators used in these methods show color changes depending upon the reaction of the medium. Each indicator changes color through a definite, limited range of H ion concentration, the shades of the indicator between these limits corresponding to definite pH values.

TABLE I. COMMON INDICATORS; THEIR pH RANGE AND COLOR CHANGES

INDICATOR		RANGE	COLOR CHANGE	
			Acid	Alkaline
Acid	{ thymol blue	1.2-2.8	Red	Yellow
	{ brom phenol blue	3.0-4.6	Yellow	Blue
	{ methyl red	4.4-6.0	Red	Yellow
Neutral	{ brom cresol purple	5.2-6.8	Yellow	Purple
	{ brom thymol blue.	6.0-7.6	Yellow ¹	Blue
Alkaline	{ phenol red	6.8-8.4	Yellow	Red
	{ cresol red	7.2-8.8	Yellow	Red
	{ thymol blue	8.0-9.6	Yellow	Blue

Brom cresol purple, brom thymol blue, and phenol red, being nearest the neutral point ($\text{pH} = 7.0$), are the indicators of special value to the bacteriologist.

In the colorimetric method a tube of the medium with the indicator added to it is matched up in the comparator block with standard tubes which have been prepared. For example, if the tube of medium plus the indicator matches the standard tube with a $\text{pH} = 8$, then the reaction of the medium is $\text{pH} = 8$. The H ion concentration of any solution can be roughly estimated by finding what indicators give their acid or alkaline color in it. An indicator may be added to the medium in which bacteria are growing to test roughly the change in H ion concentration produced by the organism.²

By far the most accurate determination of H ion concentration is by means of the potentiometer. This method is not so well adapted to elementary students; it requires elaborate apparatus and is time-consuming.

Ways of using culture media. Most culture work in the bacteriological laboratory is carried on with cultures made in bacteriological culture tubes. The standard tubes used are longer than common test tubes, have no lip, and are made of fairly thick heat-resistant glass to reduce breakage. Wide-mouth tubes, where a large surface is exposed, are of

¹ At the neutral point it is a grass green — a color which can be sharply recognized.

² For methods of preparing and adjusting culture media see Appendix B, p. 299.

great advantage when an actively motile broth culture is desired. Tubes are conveniently packed in wire baskets when sterilized and when kept in the ice box.

Liquid media are measured in approximately 10-cubic-centimeter amounts into these tubes, which are then plugged and sterilized. Fermentation tubes of the types shown in

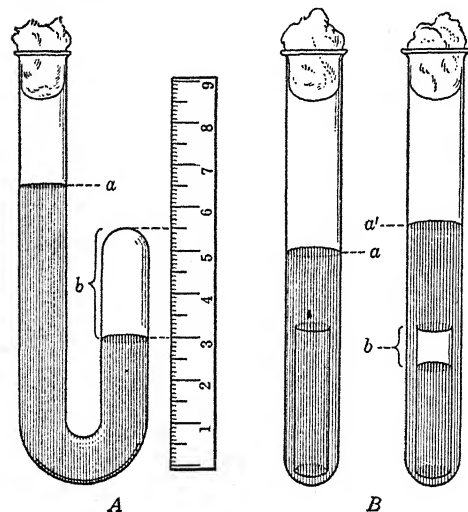


FIG. 25. Fermentation tubes

A, Smith's type, showing the method of measuring the column of gas: *a*, level of the fermenting medium in the open arm; *b*, space filled with gas resulting from fermentation and caught in the closed arm. B, Durham's type: *a*, *a'*, level of fermenting medium before and after fermentation respectively; *b*, gas generated, and collected in inverted shell vial

Fig. 25 are used for the determination of gas production in media such as lactose bile and the sugar broths. These permit a rough quantitative measurement of the gas. It will be seen in A that as gas is formed in the broth as a result of microbial activity it collects in the closed arm of the tube, displacing the broth, which as a result will rise in the other arm. Now, placing a rule against the tube and reading from the meniscus up, we can measure the column of gas which has been formed. This

method gives us a means of comparing gas production of various organisms. The other type of fermentation tube illustrated consists of an inverted shell vial in a tube of broth. During sterilization the air will be expelled, and, upon cooling, the vial will fill with the medium. As gas bubbles are formed by fermentation they will collect in the vial. Quantitative measurement may then be made of the total amount of gas trapped. Qualitative analysis of the gas may also be

made by the use of special apparatus. Agar and gelatin are usually tubed in measured amounts. The tubes may then be melted and *agar slants* made by cooling in a slanting position, or the melted medium may be poured into a Petri dish for a *plate culture*. Erlenmeyer or Florence flasks are used when larger quantities of media are desired. Large bottles with the medium slanted in them so that an extensive surface is exposed are used in the production of tuberculin.

Stab cultures are made by inoculating solid media by a deep puncture. The place of growth in the tube gives us a general idea of the relation of the organism to oxygen. Stab cultures are made in gelatin to test the liquefying (proteolytic) action of the organism.

We cannot emphasize too strongly the need of the *aseptic method* in the laboratory. The beginner cannot understand why experiments so often do not "come out right." Whatever we are doing, the greatest care must be taken to prevent contamination; the inoculating needle must be carefully sterilized before and after each operation; the mouths of tubes must be flamed before withdrawing material and upon opening a sterile tube. The cover of the Petri dish should be opened only enough to insert the mouth of the test tube when pouring an agar plate.

In making an agar plate the medium may be inoculated before pouring (having been cooled sufficiently so that the organism will not be killed), or, more commonly, measured amounts of the material to be tested may be pipetted into a plate and the melted and cooled agar poured upon this inoculum and mixed. This gives us a quantitative method for estimating the number of bacteria per cubic centimeter,



FIG. 26. Inoculating needles

a, loop for transferring a drop of liquid medium; b, straight needle for inoculating solid media

or per gram of substance. For example, one gram of garden soil may be weighed out, transferred to a sterile water blank containing 99 cc. of water, and thoroughly shaken, resulting in a 1/100 dilution of the original material. One cubic centimeter of this 1/100 dilution added to 9 cc. of sterile water gives a 1/1000 dilution, or 1 cc. of the original dilution added to 99 cc. of sterile water results in a 1/10,000 dilution. Any dilution desired can be made in this way. With a liquid sample, as water or milk, 1 cc. is pipetted into the first dilution bottle. Each dilution must be vigorously shaken to insure thorough distribution of the organisms. In all quantitative work not less than three plates should be prepared. It is well to check up occasionally on the sterility of the media and glassware being used by preparing sterile controls.

If upon examining an incubated plate of a 1/100 dilution fifty colonies are counted, then, multiplying by the dilution factor, it is estimated that there are five thousand bacteria in a gram or in a cubic centimeter of the original sample. Counts obtained from plates made from the same sample may be averaged, if they do not differ by more than 20 per cent, and this average expressed as the total number of bacteria in the samples tested. If the material is heavily contaminated and insufficient dilutions are made, not all the bacteria present will develop into colonies, owing to crowding. Careless technique, such as exposure of plates to the dust of the room, insufficient shaking of the diluted samples, inaccurate weighing and pipetting, and errors in counting, all contribute as sources of error in the experiment. The number of colonies is always less than the actual number present, because (1) clumps of cells develop into one colony; (2) the medium, incubation temperature, or other culture conditions may not be appropriate; (3) the bacteria may be of low vitality or dead.

In recording counts, figures which give a fictitious accuracy are dropped, and the number of colonies, which is a measure of the number of bacteria, per cubic centimeter or per gram is recorded as shown in the table on the following page.¹

¹Standard Methods of Water Analysis. 1936.

TABLE II. NUMBER OF BACTERIA PER CUBIC CENTIMETER

From	1 to	50 shall be recorded as found	
From	51 to	100 shall be recorded to the nearest	5
From	101 to	250 shall be recorded to the nearest	10
From	251 to	500 shall be recorded to the nearest	25
From	501 to	1,000 shall be recorded to the nearest	50
From	1,001 to	10,000 shall be recorded to the nearest	100
From	10,001 to	50,000 shall be recorded to the nearest	500
From	50,001 to	100,000 shall be recorded to the nearest	1,000
From	100,001 to	500,000 shall be recorded to the nearest	10,000
From	500,001 to	1,000,000 shall be recorded to the nearest	50,000
From	1,000,001 to	10,000,000 shall be recorded to the nearest	100,000

The spreading of growth on plates, due to the condensation of moisture, is prevented if the plates are inverted in the incubator. The medium then gradually absorbs the moisture.

Methods of transferring and cultivating microorganisms. In making bacterial cultures the material is inoculated by means of platinum needles made by fusing wire into the end of glass tubing.

A colony from an agar plate may be touched, or *fished*,

with the straight needle and transferred to the medium in which it is to be grown; or a loopful of a broth culture may be transferred to another tube of sterile broth in making a *subculture*. In all operations the greatest care must be taken to prevent contamination from the outside. The process of transferring a culture involves the opening of the tube, the removal of the material, and the inoculating of the new medium. The student must practice the correct handling of

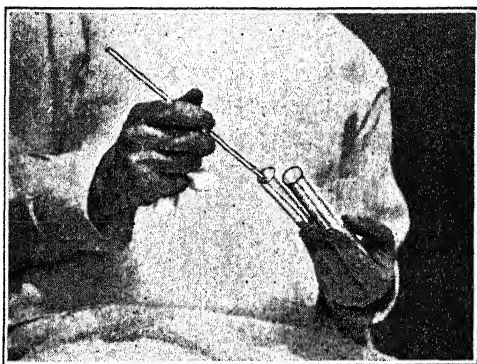


FIG. 27. Transferring a culture from one tube to another

Note the way the needle, test tubes, and two cotton plugs are held

tubes, needle, and plugs in making transfers. It will require time and patience, but only by the mastery of this technique can he hope for accurate bacteriological work. The technique which the elementary student has attained must be perfect, so that there is no possible danger to himself or his neighbor when, later, he works with disease-producing organisms.

Methods of isolation of microorganisms in pure culture. To study an organism satisfactorily demands the isolation of the organism in *pure culture* — a culture in which there is only one species of organism present.

Isolation by means of direct transfer. The ideal procedure would be to select one cell and transfer it to a nutrient medium. The growth resulting would then be a pure culture. Bacterial cells are so minute that it requires a very special and difficult technique to isolate one individual cell. This technique, however, has been satisfactorily accomplished by Barber by means of capillary pipettes, and an elaborate instrument which picks out a single organism under the microscope.

Dilution. The first method to be employed by the early investigators in making pure cultures was by dilution. If, in several tubes containing the material in very high dilutions, growth was obtained in only one of them, it was inferred that the growth arose from one remaining cell. It is obvious that the method is crude and ill adapted to our use today.

Isolation from colonies in plate cultures. Selection of colonies from plate cultures is a more reliable method and one most commonly used in the laboratory. We have already seen the importance of solid media in pure-culture work and the use of plate cultures. The cells remain isolated when the medium solidifies, and give rise to the formation of colonies, each colony having arisen, presumably, from the growth of a single cell. Selection and fishing from any colony would then give us a pure culture of an organism. The material to be plated must be diluted sufficiently; otherwise the colonies will grow too close together to be readily isolated.

Streaking. If a mixed culture is smeared over the surface of a solid medium in successive streaks, discrete colonies will

develop where the material is thinned out by streaking. It is then possible to select a colony and transfer to another medium.

Animal inoculation. Animals may be made use of to obtain a pure culture of an organism. If a mixed culture containing a disease-producing organism (pathogen) is inoculated into a susceptible animal, the organism causing the disease will thrive, while the other forms fail to grow, owing to the antiseptic action of the body fluids. The pathogenic organism may then be isolated in pure culture from the tissues when the animal is autopsied.

Isolation by heat and chemicals. Material containing resistant spores may be heated to a temperature high enough to kill all vegetative cells (80° C. for thirty minutes) and then plated out. The colonies which develop arise from the germinating spores.

A selective antiseptic may be used which will inhibit the growth of all but a single form. Bile is helpful in the isolation of intestinal bacteria from polluted water because it commonly inhibits the growth of organisms other than those found in the intestinal tract. The addition of a dye to the medium is often of value in pure-culture work. The colonies which develop on the surface of the dye medium may then differ in color and in this way facilitate the selection.

The cultivation of anaërobic microörganisms. The two groups of bacteria, the aërobes and the anaërobes, call for very different cultural methods. All methods for making anaërobic cultures must provide in some way for the removal of oxygen. Cultures may be grown in vacuo; the oxygen may be replaced by some inert gas; or it may be absorbed by some chemical reaction.

Pasteur poured sterile oil over the surface of the medium, in this way excluding the atmospheric oxygen. Liborius first used stab cultures, planting the organism deep down in the medium.

Hydrogen is ordinarily used in replacing the oxygen of the atmosphere, as it has no injurious effect on the organisms.

The tubes are placed in a Novy jar into which hydrogen is introduced from a Kipp generator. There are several methods for absorption by the use of chemicals; the pyrogallic acid method is the one most commonly used in the laboratory. Tubes of dextrose agar are boiled gently over the free flame

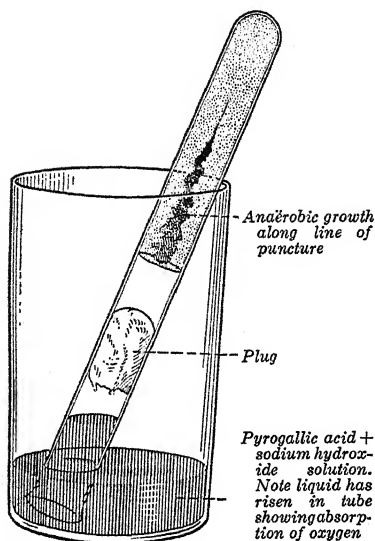


FIG. 28. A simple method for making an anaerobic culture of bacteria on solid media

to expel the oxygen, and are allowed to cool in a slanting position; the slanted surfaces are then inoculated with the culture. The cotton plugs are cut flush with the edge of the tubes and pushed in about two inches from the top. The tubes are then inverted and incubated in a jar which contains a mixture of pyrogallic acid and sodium hydroxide solution. The liquid will rise in the tubes to replace the oxygen absorbed. Care must be taken not to disturb the tubes, lest we admit air.

The oxygen may be abstracted by the use of a mercury or water pump and the organisms cultivated in a vacuum. A method for the removal of oxygen by reacting with hydrogen in the presence of platinized asbestos has been described.¹

Microscopic examination of bacteria. After an organism has been obtained in pure culture we come to the study of cell structure which involves the use of the microscope. To appreciate what a wonderful mechanism we have in a compound microscope the student should compare the picture of the simple instruments used by early microscopists with the

¹ Richardson and Dozler, in *Journal of Infectious Diseases*, Vol. XXXI (1922), p. 617.

present-day instrument (Figs. 1 and 2). The art of grinding lenses sufficiently fine to reveal these minute forms of life has been gradually perfected, and methods of illumination have been devised to give us the instrument which we use today. Koch was the first to employ devices such as the Abbé condenser and the oil-immersion lens in the study of bacteria.

Unstained preparations. In studying living organisms the hanging-drop preparation may be used. The edge of the depression in a hollow-ground slide is outlined with vaseline. Then place a drop of the culture in the center of a clean cover slip and invert the hollow side over it so that the cover adheres to the vaseline. When the slide is turned over, the cover slip is on top and the drop is suspended from it in this air-tight chamber. The organisms, being transparent, will not be seen distinctly if the lower iris diaphragm on the condenser is not partly closed. The student should focus first on the edge of the drop. The hanging-drop preparation is of special value in determining the motility of organisms and the effect of various agents on living cells, particularly the reaction to serums. Motility must not be confused with the Brownian movement, which is exhibited by all minute particles in suspension.

Fresh, unstained material is more readily visible when studied under the *dark-field microscope* or *ultramicroscope*. The transparent particles are then brightly illuminated against a dark background. Flagella may also be demonstrated under the dark-field microscope in a suitably prepared mount.

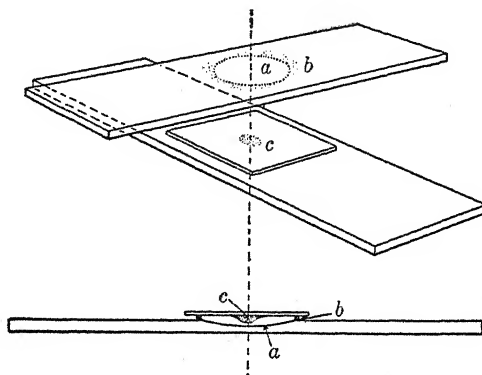


FIG. 29. Hanging-drop preparation

a, depression in slide; b, film of vaseline around the rim; c, small drop of culture to be examined

Stained preparations. The early investigators realized that the motility of organisms, and the fact that they were colorless and transparent, prevented their accurate study. The development of microscopic technique involving proper illumination and staining methods has overcome these difficulties. Koch saw that if the bacteria could be killed, fastened (or *fixed*) to the slide, and stained, they could be more accurately studied. In 1877 he devised a simple method of staining. This opened up a new field and accelerated the study of morphology, which years of laborious study of unstained preparations had not appreciably advanced.

Most bacteriological stains are made of anilin dyes. There are a large number of dyes for special purposes, but the elementary student will rarely use any except fuchsin, methylene blue, gentian violet, and some that are used as counterstains, such as safranin, eosin, and Bismarck brown. The phenomenon of staining is essentially a chemical one, the protoplasm of the cell combining with the dye substances. Different parts of the cell react differently to stains, thus giving a means of bringing out certain cell structures. The bacterial spore resists staining, so that in order to dye it the penetrating power of the stain must be increased by heating or by prolonging the process. The spore then retains the color when washed in alcohol. Special stains are used to bring out metachromatic granules within the cell, capsule formation about the cell, and the presence of flagella. The latter is one of the most difficult of procedures.

The general technique of fixing and staining is as follows:

1. Remove a loopful of the material to be examined, and spread it evenly in the center of a clean microscopic slide. If examination is to be made of growth from solid media, a drop of distilled water is put on the slide and the inoculating needle washed off in this.
2. Dry *slowly*, either naturally or by holding the slide by forceps well above the gas flame.
3. *Fix* material to the slide by passing through the flame three times.

4. Apply the stain, leaving it on for the required length of time. Several stains acting as mordants, or decolorizers such as alcohol, may be used here.

5. Wash off the stain.

6. Dry by shaking off all excess water and by blotting or blowing across the surface of the slide. The slide may be very carefully warmed to hasten drying.

7. Put on a drop of immersion oil.

8. Examine with the oil-immersion objective.

Methylene blue is the most commonly used and undoubtedly the first stain which the student will use in the laboratory. Sometimes certain substances are added to the solution, which increase the staining power by fixing the dye in the cell. Such substances are known as mordants. The alkali in Löffler's methylene blue acts in this way. Carbolic acid acts as a mordant and, combined with heating during the process of staining, thoroughly fixes the dye to the bacterial cells.

The preparation of a few of the more common stains and the technique of their use follows:

LÖFFLER'S METHYLENE BLUE

Saturated solution methylene blue in alcohol	30 cc.
Solution potassium hydroxide in distilled water (1:10,000) . . .	100 cc.

Technique of the Löffler stain. Flood the fixed smear with the methylene blue stain, and let stand for fifteen to thirty seconds. Wash off the stain and dry.

ZIEHL-NEELSON CARBOL FUCHSIN STAIN

Basic fuchsin	1 gm.
Ethyl alcohol	10 cc.
5 per cent solution carbolic acid	100 cc.

Technique of the Ziehl-Neelson (acid-fast) stain. 1. Flood the smear with carbol fuchsin.

2. Holding the slide with the forceps, heat *gently* over the flame until steam begins to rise. Do not allow the stain to evaporate to dryness. Steam from two to five minutes.

3. Wash and decolorize with acid alcohol (2 per cent solution of hydrochloric acid in 95 per cent alcohol). Decolorize until no more stain comes out.

4. Wash in water.

5. Counterstain for a few seconds with methylene blue.

6. Wash and dry.

Because of the lipoid nature of the cell wall the tubercle bacillus is stained with considerable difficulty. It is therefore necessary to heat the stain in order that it may penetrate. When once stained, however, the cells resist decolorizing even in acid alcohol. Body cells as well as other organisms give up the original stain and subsequently take the color of the counterstain. The *acid-fast* organisms stand out sharply, stained bright red against the blue background.

CARBOL GENTIAN VIOLET

Gentian violet (saturated alcoholic solution)	10 cc.
Carbolic acid	1 gm.
Water	90 cc.

GRAM'S IODINE SOLUTION

Iodine	4 gm.
Potassium iodide	8 gm.

Dissolve in 20 cc. distilled water and then add 1200 cc. water.

Technique of the Gram stain. 1. Fix and dry the smear in the usual manner.

2. Flood the smear with the gentian violet and leave on for one and one-half minutes.

3. Wash in water.

4. Treat with Gram's iodine solution for one minute.

5. Wash in water.

6. Decolorize with 95 per cent alcohol for two minutes.

7. Wash in water.

8. Counterstain with dilute fuchsin or other contrast stain, as safranin, for about thirty seconds.

Gram's method of staining is an important means of differentiating bacteria into two groups, the *Gram-negative* and the *Gram-positive*. Some bacterial cells, when treated with the

gentian violet stain and iodine, retain the dye after treating with alcohol, while others do not. The Gram-positive bacteria possess this property and therefore will appear purplish black at the completion of the staining, while the Gram-negative cells, lacking this, appear pink or brown or some other differential color, depending on the counterstain used. Below is a list of some of the most important Gram-positive and Gram-negative organisms, including certain pathogens in which the Gram technique is of diagnostic importance.

GRAM-POSITIVE

Streptococcus pyogenes
Streptococcus hæmolyticus
Staphylococcus aureus
Diplococcus pneumoniae
Bacillus subtilis
Bacillus anthracis
Clostridium tetani
Corynebacterium diphtheriae

GRAM-NEGATIVE

Encapsulatus pneumoniae
 (Friedländer)
Neisseria intracellularis
Neisseria gonorrhoeae
Escherichia coli
Eberthella typhi
Eberthella dysenteriae
Proteus vulgaris

Animal inoculation. The use of animals is indispensable in fostering scientific progress and in routine bacteriological work. The rôle this has played in the advancement of science is tremendous; the results are the saving of untold human lives.

Laboratory animals may be used in the following ways:

1. For the diagnosis of disease.
2. For the production of vaccines and sera.
3. To test and standardize biological products.
4. For experimental work.

In the first place, it is necessary to choose an animal which is susceptible to the specific pathogenic organisms if a disease is to be produced experimentally. Guinea pigs, rabbits, and white mice are most commonly used for the study of diseases peculiar to man. Horses or goats are employed for the production of diphtheria, tetanus, and other antitoxins; calves produce the virus which is used in smallpox vaccination. Animals are used to check the potency of biological products and to standardize these products for our use. Often diagnosis

of disease could not be made without animal inoculation. A survey of the history of medicine will reveal to the student the importance of animals in determining the causative organisms of a disease. *Koch's postulates* to prove an organism the etiological agent in a disease are :

1. The specific organism must always be found associated with the disease.
2. This organism must be isolated in pure culture.
3. When inoculated into a healthy susceptible animal it must produce the characteristic symptoms of the disease.
4. The organism must finally be recovered from the animal in pure culture.

Material to be inoculated into an animal is drawn into a sterile hypodermic syringe and injected. Inoculations may be made subcutaneously (under the skin), intradermally (between the layers of the skin), intravenously (into the veins), or intraperitoneally (into the peritoneum).

Occasionally we resort to certain serum reactions to prove the identity of an organism. These are discussed in the last chapter of the book. The reaction in any case depends upon the presence of chemical substances produced by the body during the course of the disease.

CHAPTER VII

THE NUTRITION AND RESPIRATION OF THE BACTERIA

The underlying principles of the physiology of the bacteria are paralleled, in most instances, in the more highly organized plants and animals. However, we find two unique activities of bacteria: some thrive in the absence of free atmospheric oxygen; others are capable of oxidizing free nitrogen gas. It may be profitable to review hastily the general principles of plant and animal nutrition and respiration.

The *chlorophyll-bearing* (or green) plants, in the sum total of their activities, may be spoken of as *synthetic* organisms. They use inorganic substances, carbon dioxide from the air and various salts from the soil, and by absorbing or filtering out the radiant energy of the sunlight they bring about the combination of the simpler chemical elements into the more complex organic compounds. The sum total of plant activity is therefore the storing of energy, radiated from the sun, in organic chemical substances, as sugar and starch, cellulose, and plant proteins. Accompanying these constructive (anabolic) changes there are certain destructive (katabolic) processes continuously going on. For the most part these are oxidations, or slow burning of the stored material, in the living tissues; and therefore plants are constantly taking in oxygen and giving off the chief waste products of combustion, CO_2 and H_2O . In other words, plants respire and require a continuous supply of atmospheric oxygen. All plants require water or moisture, for part of their food at least must be taken in soluble form from the soil or from the medium which surrounds them.

Animals represent, in their sum total of life activities, destructive or *analytic* changes. They are solely dependent upon the green plants for energy and building foods, and

although highly complex substances are synthesized in the animal body, the balance is excessively in favor of the destructive processes. Animal energy is obtained chiefly from the combustion or oxidation of organic matter, and a constant abundant supply of atmospheric oxygen is ever essential. Moisture is absolutely necessary to animals, either (in the case of lower forms) to permit direct exchanges with environment or else to act as the transporting medium for foods, gases, and wastes within the body.

The life of the plant or animal will be healthy and normal or unhealthy and abnormal, according to the inheritance with which it is endowed and the environment within which it lives. The multiplication of the species will depend upon natural endowments and the condition of the surroundings, and so the rate of increase or decrease will depend upon the balance of birth and death rates.

Bacteria in their metabolism for the most part resemble animals more closely than they do the green plants. With a few exceptions we find their destructive chemical actions in excess of their building-up processes. They live for the most part upon organic matter, though in many cases this is subject to great modification. Some bacteria are even capable of using simple inorganic salts and gases to build up their own complex living substance.

Bacteria are much more independent of their *oxygen relations* than either plants or animals. The almost unbelievable phenomenon of life without free oxygen is possible for many bacteria. Others find most favorable conditions with a partial oxygen tension or with ordinary atmospheric oxygen, and some are very indifferent within wide limits as to what the oxygen concentration is.

For their life activities all bacteria are absolutely dependent upon free or condensed water. They put themselves in relation with their surroundings only through the intermediation of the cell membrane, and solid dry matter is useless to them.

Finally, their origin is always from previous representatives of the race, and not spontaneous; they are endowed from one

generation to the next with inherent, hereditary possibilities; they enjoy a good birthright or a poor one according to their ancestry. The rapidity of birth or reproduction will depend, as with other organisms, upon this hereditary factor and upon the condition of the surroundings; and the longevity and death rate will also be modified by these factors. Bacteria do mature, and, if not actively reproducing, grow old and die. It is fundamental to think of bacteria in these biological terms and not as something extraordinary and different from other living things.

Physiology of the cell. The physiology of the one-celled organism is primitive in that there can be no differentiation of function. The cell is self-sufficient, providing the secretions which digest the food, absorbing the digested products, ex-

creting the wastes or by-products of metabolism, and being endowed with the power to reproduce. When we think of such a speck of matter endowed with these remarkable properties, the one-celled organism becomes surprisingly complex. The cell membrane relates the organism to the immediate external environment. We have seen that the passage of water inward or outward is determined by the relative concentration of salts or sugars within the cell and in the fluid medium surrounding it. Under ordinary conditions the passage of food for energy, maintenance, and growth, as well as the discharge of waste matter from the cell, must depend upon a selective process.

The passage of molecules through membranes is determined by the nature of the membranes and the state of the

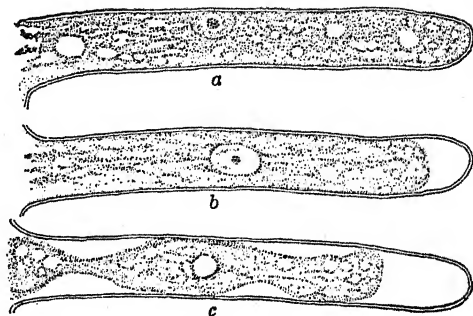


FIG. 30. Plasmolysis in root hairs from root of a mustard seedling

a, natural, turgid state; *b*, beginning of plasmolysis; *c*, later stage of plasmolysis (owing to the removal of water, the protoplasm has shrunk from the cell wall, but the wall itself remains turgid)

molecules. *Semipermeable* membranes allow the passage of water through them, but do not permit solutes to pass. *Dialyzing* membranes allow salts and sugars to pass according to the relative concentration of the solutes on either side of the membrane; dialysis always takes place from the solution of greater to that of lesser concentration, while water passes in both directions until equilibrium is established. Substances like egg albumin or gelatin are not dialyzable.

Crystalloids and colloids. In general we may divide matter in solution into two categories according to its physical state. (1) *Crystalloids* go into a true, or ionic, solution uniformly dispersed throughout the solute. The molecule in solution may or may not undergo electric dissociation. Cane sugar does not so dissociate, while sodium chloride breaks up into ions of sodium and chlorine bearing positive and negative charges of electricity respectively. Sugar is called a nonelectrolyte; salt, an electrolyte. Ionization increases the number of free particles in solution, and their ionized state makes them more effective as regards the pressure or tension that they exert on membranes.

(2) *Colloids* do not go into a true solution and are not uniformly dispersed. They are not ionized, although they respond to an electric current. Their molecules are relatively very massive, and their relation to membranes is inert. They are represented by nitrogenous molecules, like casein in milk, egg albumin, or a solution of gelatin in water. Their filtration through artificially prepared membranes may be controlled by the size of the pores in the membrane. As we have seen, they do not commonly pass through dialyzing membranes.

The surrounding membrane of the bacteria, including the highly specialized layer of cytoplasm immediately inside the defining wall, separates the complex living matter from the environment. Outside the membrane is the water which holds in solution the potential foods (salts, carbohydrates, fats, and proteins) as well as various gases. Some of these substances may be in available form to serve the organism as food directly; most of them must be acted upon and split or

broken into simpler chemical compounds before they can diffuse or be absorbed by the cell and thus become available for energy or building material. The nature of the absorption process is not well understood, but it is known to be highly selective, and for lack of knowledge of its true physics and chemistry we may speak of a "vital force" being involved. The preparatory splitting action, or digestion, is carried on by ferments, or enzymes, originating in the cells and being secreted through the surrounding membrane. Scientists confess ignorance of exact knowledge of the chemical nature of enzymes or of the process of secretion. Enzymes are colloidal in nature, being therefore very large complex molecules made up of several elements.

On the inner side of the membrane is the living substance, protoplasm. The vital processes themselves occur here: the synthesis of the absorbed food into living substance; the formation of waste products which must be excreted through the membrane; and the manufacture of the enzymes — both those to be secreted (the extracellular) and those retained within the cell for its functioning (the intracellular). Broadly speaking, they carry out, respectively, the two chief types of cell activity — digestion (the analytical process) and maintenance and growth (the synthetic process).

Chemical composition of the bacteria. The exact chemical composition of living matter is not known. What the dead matter in the living body contains is susceptible of analysis. Massive cultures of bacteria may be grown and freed from the substratum for analysis. When the organic matter is eliminated by combustion, the remaining ash shows a surprising proportion of phosphorus (phosphates), often representing 50 per cent of the total. Potassium, sulphur, chlorine, and calcium are often present in large proportions, while traces are found of a number of other elements. The ash constitutes from 5 to 30 per cent of the total dry cell-substance. The table ¹ on the following page illustrates the general chemical composition of certain bacteria.

¹ A. I. Kendall, *Bacteriology*, p. 58.

TABLE III. PERCENTAGE COMPOSITION WITH RESPECT TO ORGANIC AND INORGANIC CONSTITUENTS

	PUTREFACTIVE BACTERIA	SERRATIA MARCESCENS	MYCOBACTERIUM TUBERCULOSIS
Water	83.42	85.45	85.00
Protein	13.96	10.33	8.50
Extractive	1.00	0.70	4.00
Ash	0.78	1.75	1.40
Residue	0.84	1.77	1.10

Carbohydrates and fats are commonly found in bacteria. Glycogen or a similar substance is the usual form of carbohydrate. Fats, often apparently contained in the cell wall, are sometimes present in large amounts — as high as 40 per cent of the dry residue in tubercle bacilli, for example.

The protein substances constitute from 50 to 75 per cent of the total dried substance. The amino acids yielded by different bacteria are fundamentally different, as shown by the following table:¹

TABLE IV. PERCENTAGE OF DIFFERENT AMINO ACIDS FOUND IN COLON AND TUBERCLE BACILLI

	COLON	TUBERCLE
Glutamic acid	3.00	0.20
Glycocoll33	0.00
Alanin	1.00	1.40
Valin	1.60	4.60
Leucin	2.00	1.82
Phenylalanin	0.20	0.50

The typical cell wall of higher plants is composed chiefly of cellulose. While cellulose has apparently been identified in certain of the higher bacteria, it is not common. Chitin, which is a typical animal product found in the shell of various arthropods, has been reported to be present in the bacterial cell wall. It contains the amine radical NH_2 and is a polymer of glucosamine.

¹ V. C. Vaughan, Protein Split Products, p. 88.

It has already been noted that the capsules of bacteria are composed of highly complex polysaccharides, which play an important rôle in determining their disease-producing powers and determine their specific antigenic properties. These appear to be present in all bacteria so far studied.

In certain bacteria, as the sulphur or iron bacteria, we naturally find a high proportion of those elements which are essential to their metabolism.

The food of bacteria. Bacteria thrive under most diverse conditions. Some bacteria are highly discriminating in their food requirements, while others exhibit a wide range of choice. Parasitic bacteria generally require special food substances as well as a carefully adjusted physical environment. This is also true of the nitrogen-fixing bacteria of the soil. The ordinary bacteria of decomposition, on the other hand, flourish indiscriminately on any pabulum rich in dead organic matter.

On the basis of nutrition we may conveniently divide the bacteria into three groups:¹ (1) The *prototrophic* (autotrophic) bacteria are those requiring no organic compounds or only traces of carbohydrates for their nutrition. (2) The *metatrophic* (heterotrophic) bacteria thrive under a wide variety of conditions where there is water and organic food. The vast majority belong to this group. (3) The *paratrophic* bacteria live as parasites in the living tissues and fluids of the plant or animal host. They rarely thrive in the external environment, and many are grown with difficulty in the laboratory.

It is rare in nature, except with the pathogens, to find bacteria in pure culture. In the soil, in water, or in milk we may find many different kinds of bacteria intimately associated, and we must assume either a keen competition for food or perhaps a state of helpfulness, where the changes induced by one organism may be an essential preparatory process for others which grow secondarily. Laboratory experiments rarely approximate the conditions under which bacteria grow in nature.

The uses of food for microorganisms are the same as for higher forms of life: for energy purposes, to make possible

¹ A. Fischer, *The Structure and Functions of Bacteria*.

movement, circulation of the protoplasm, etc.; for the incorporation of inert chemicals into the living state and for growth; and for reproduction, which in the bacteria is ordinarily a direct result of growth.

The amount of food required by a single bacterium is infinitesimal. Some bacteria will live and multiply to considerable numbers in the purest distilled water which the chemist can produce. The maximum amount of food that bacteria may use is infinitely great. The maximum rate of multiplication never continues for any appreciable length of time but is limited by natural causes, the most important being the accumulation of their own waste products and the exhaustion of essential food elements.

When we consider the qualitative aspects of food supply for the bacteria we find that within the broad groups of prototrophic, metatrophic, and paratrophic organisms there are very wide variations. Traces of inorganic salts containing the elements found in the ash are essential to all bacteria. We have seen that some specialize in the metabolism of one special element, as iron or sulphur. Excluding the prototrophic bacteria, all require organic food, carbohydrates, and proteins, while some attack fats. The carbon compounds serve essentially as energy or fuel foods; the nitrogen compounds serve the purpose of growth.

The availability of carbohydrate foods depends chiefly upon the size of the molecule, but also upon the construction, or geometric configuration, of the molecule. It follows that the monosaccharides are fermented with the greatest avidity; then, in order, the disaccharides, trisaccharides, and polysaccharides (starches). The glucosides and certain alcohols may also serve bacteria as sources of carbon and thus fit into the "metabolic gradient." The following table, taken from one of the author's works on two hundred and forty-two pure cultures of streptococci, shows the readiness with which seven representative substances are fermented:¹

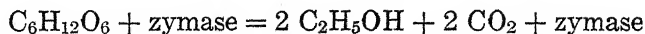
¹ C. M. Hilliard, in *Journal of Infectious Diseases*, Vol. XII, No. 2.

	PER CENT
Glucose ($C_6H_{12}O_6$), a monosaccharide	98.0
Lactose ($C_{12}H_{22}O_{11}$), a disaccharide	76.0
Saccharose ($C_{12}H_{22}O_{11}$), a disaccharide	65.5
Salicin ($C_{13}H_{18}O_7$), a glucoside	42.7
Raffinose ($C_{18}H_{32}O_{16}$), a trisaccharide	37.5
Inulin ($C_6H_{10}O_5$) _n , a starch	9.0
Mannite ($C_6H_{14}O_6$), an alcohol	1.5

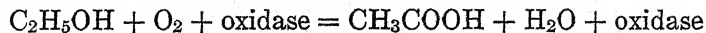
It will be noted that while lactose and saccharose have the same molecular weight, their availability is quite different. With most bacteria, and especially with yeasts, this order is reversed, lactose being less commonly attacked. This discrepancy must be explained by the stereochemical structure, and it may be that the aldehyde group in lactose renders it harder to split.

Fats and oils are relatively resistant to destruction by bacterial action, as we know from our practical experience in the home with foods like lard and other cooking fats. This is due partly to the low water content of such products but also to the fact that but few bacteria produce fat-splitting enzymes (lipase). None are wholly immune to bacterial decomposition and may be hydrolyzed into glycerin and fatty acids. Organic acids, as lactic, may likewise serve as energy food for some microorganisms.

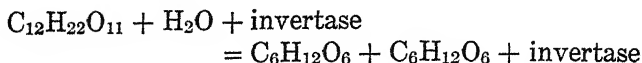
As has been stated, the primary service of the carbon foods is to yield energy. Chemical compounds are split, or rended apart, to form two or more new compounds, and thereby energy is released. The enzymes chiefly involved are splitting enzymes (zymases) which act without the addition of either water or oxygen. The splitting of glucose into ethyl alcohol and carbon dioxide is an example:



Carbohydrates may, however, be oxidized in a way analogous to the formation of acetic acid from alcohol:

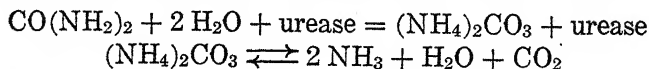


Again, hydrolysis may occur as in the case of cane sugar, which splits into glucose and fructose:



The availability of the nitrogenous compounds is quite the opposite of what has been described for carbohydrates. For the majority of bacteria the massive protein molecules, made up of hundreds of atoms, serve as the readiest source of food. Proteins, upon being digested or split, break up into amino acids, of which about twenty are known. The bacterial protoplasm is made up of combinations of several of these amino acids variously put together. One organism requires an excess of one acid; another, a different one. The table on page 84 shows the differences in amino acid analysis of colon and tubercle organisms. To find the right pieces to fit into this mosaic it is evident that the wider the choice the greater the probability of success.

Next in order of usefulness to the proteins come the albumoses, polypeptids, peptones, free amino acids, and ammonia compounds. This is the order in which higher nitrogenous molecules are disintegrated, and one form may split off the chemicals which will serve another as food. The splitting of the higher nitrogenous compounds is usually due to hydrolysis or to the addition of water. The simplest illustration that can be given is the conversion of urea into ammonia:



This particular reaction is of greatest importance, as it will be remembered that the chief nitrogenous excretory product of animals is urea. Bacteria never form this compound.

Ammonia salts serve one group of the prototrophic bacteria as sources of nitrogen, and the nitrous salts which they form are further oxidized by another group into nitrates.

In addition to the foods discussed certain bacteria amaze us by their use of substances that we do not ordinarily think

of as serving the purpose of food, as marsh gas, carbon monoxide, formic acid, or certain thiosulphates. Thus, while the general laws of metabolism for animals hold for most bacteria, there are exceptions which break all rules and make us realize that an explanation of life itself is made more complicated as we contemplate the bacteria.

Respiration. We commonly think of free oxygen as being essential to all life. Animals and plants, both aerial and aquatic, require oxygen in the surrounding medium, and if the supply is cut off they rapidly die.

The bacteria as a group vary radically in their oxygen requirements. They may conveniently be divided into four

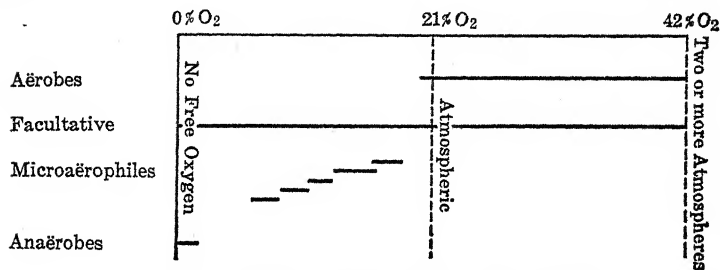


FIG. 31. Relation of bacteria to oxygen tension

quite distinct groups: (1) obligate aërobes, which require abundant free oxygen for their life activities; (2) obligate anaërobes, which are remarkable in that they grow in the almost complete absence of oxygen; (3) facultative bacteria, which are rather indifferent in their oxygen requirements and thrive in either the presence or the absence of oxygen; (4) microaërophiles, which exhibit a delicate adjustment to oxygen and grow only within a narrow zone of oxygen tension.

The *aërobes* include many of the common bacteria found in the air, water, and soil. Most of the chromogenic bacteria are aërobic. A few of the pathogenic forms, notably the diphtheria and tubercle bacilli, require a supply of free oxygen.

Aërobic bacteria naturally grow best on the surface of culture media. In liquid media they form a film, or membrane, called a pellicle. If the oxygen tension is increased by

replacing nitrogen gas with pure oxygen or by increasing the atmospheric pressure, these bacteria are not greatly affected.

The *anaërobes*¹ are remarkable exceptions to living things in general. When Pasteur first described these organisms (1861) the announcement was received with incredulity. Life without oxygen was the antithesis of all that had been taught and believed up to that time! Yet there is no question of the phenomenon today, and we know not only that certain bacteria thrive in the absence of free oxygen, but that the presence of appreciable amounts of this gas will prove injurious to them. Anaërobes ordinarily thrive best in the presence of readily fermentable substances, such as sugars, from which they may derive energy.

Anaërobes may be found in the soil. Many of the putrefactive forms, such as those found in sewage, are anaërobic. Some of the pathogens (notably the tetanus bacillus, causing lockjaw) belong to this group.

The *facultative* bacteria are able to adapt themselves to a wide variation in oxygen tension, some thriving alike in the presence or absence of oxygen. Usually they grow best under one condition or the other. Some anaërobes may be adapted to increasing amounts of oxygen when gradually acclimated under experimental conditions.

The *microaërophiles* are an interesting group which grow only through a narrow zone of oxygen tension intermediate between the usual atmospheric concentration and total anaërobiosis. If a deep, narrow test tube is two-thirds filled with a liquid or semisolid medium, and a bit of sterile tissue (kidney) is added, we get all gradations of oxygen tension at different levels from the atmospheric condition at the top to total anaërobic relations at the bottom. Organisms planted in such a tube will grow most luxuriantly in the region of most favorable oxygen relations, often inhabiting a very narrow zone. Many of the parasitic organisms belong to the group of microaërophiles. The group in general offers a fruitful field for further study.

¹ Some yeasts are anaërobic.

CHAPTER VIII

BACTERIA AND THE PHYSICAL ENVIRONMENT

The life activities of bacteria, as with all living things, are conditioned by their environment. Any appreciable change in the environment, either physical or chemical, will show a corresponding change in the physiological or morphological state of the organism. We differentiate between living and dead matter by the ability of the former to adjust itself to changes in the external world. We shall find evidences of adjustment and acclimatization to new conditions exhibited by the bacteria. By an understanding of the physical, chemical, and other factors controlling growth and survival the bacteriologist is able to favor microorganisms, to suppress them, or to bring about their destruction.

A single bacterial cell, after reaching its full size, divides by fission to form two daughter cells. These attain their mature size and then divide. We can judge the effect of any physical or chemical agent upon the growth of bacteria by noting any alteration in the time between consecutive cell divisions. It is difficult to watch single cells under the microscope and in this way determine the length of time required for generation. There are methods, however, which enable us to determine the number of bacteria in a given volume of liquid. From this, calculations can be made as to the *rate of growth*.

That the student may get a conception of the growth of microorganisms, let us compare a flask of yeast cells with the population of any American city. We can, as with human beings, obtain periodic census reports by counting the number of cells present after varying periods of time, and by this we can determine the increase of the population of yeast cells.

Studying the curve shown on the following page, we see that as the cells begin to divide, growth proceeds slowly at first

and reaches the maximum where the curve approaches a vertical line (indicating here optimum conditions for growth); then the curve gradually flattens out as the rate of growth is retarded. From data obtained from other experiments we know that beyond the point plotted in this curve the line falls again, first gradually, and then with acceleration, further multiplication being prevented by external conditions such as the accumulation of waste products, insufficient food, etc.

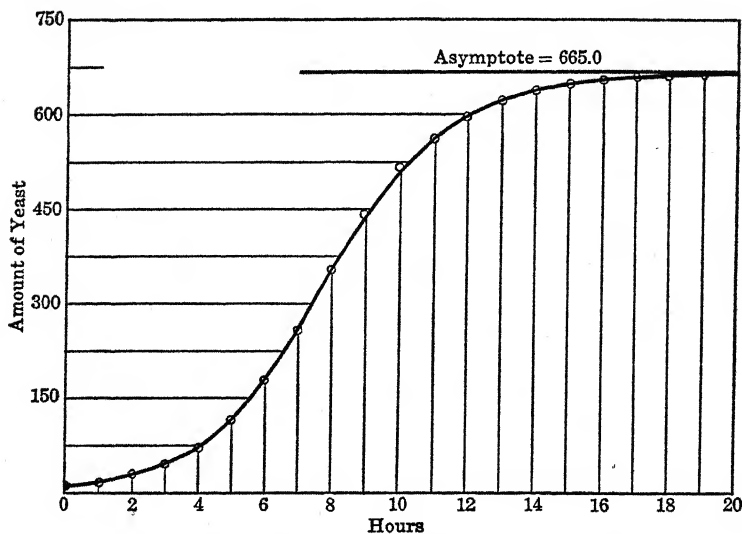


FIG. 32. The growth of a population of yeast cells¹

What is remarkable is that when Pearl comes to the study of human populations he finds that "the smooth curve is the graph of a mathematical equation of precisely the same sort as that which has been seen to describe population growth in the case of yeast."

Any unfavorable condition, if sufficiently intense, will cause bacteria to stop multiplying and to die. We are concerned with those conditions which are either inhibitory or destructive to microbial growth. The former are called antiseptics,

¹ R. Pearl, *The Biology of Population Growth*, p. 9.

and the latter disinfectants, whether the agent be physical or chemical. This distinction between antiseptics and disinfectants is an arbitrary one. It is obvious that an antiseptic under some conditions may have a lethal effect upon bacteria. A chemical ordinarily toxic may have only a mild antiseptic action if sufficiently diluted.

When exposed to the action of any disinfectant, the bacteria do not all die instantly, but the death rate follows a definite law. If the number of bacteria surviving after successive constant periods of time is determined, we find that the bacteria that have been killed bear a constant ratio to those that are living at the beginning of each period. For example, let us assume that a certain destructive agent, as heat, is applied to a pure culture containing 1000 bacteria per cubic centimeter, and we determine the surviving organisms at intervals of one minute. After the first minute, if we find one half the bacteria, or 500, to survive, then, theoretically, after the second minute one half of these 500, or 250 only, will survive, and so on in each succeeding minute until the numbers become so few that the culture is for practical purposes sterile.

It has been pointed out (by H. Chick) that the rate of death described for bacteria is the same as the rate of a monomolecular chemical reaction; that is, one where the rate of reaction is determined by the concentration of only one of the reacting molecules. The bacteria may be considered as representing such chemical molecules.

From the preceding example we see that the number of cells destroyed after a constant increment of time is always proportional to the number of cells surviving. The velocity of the reaction between the agent and the organism is known as the velocity constant. Theoretically, the figure expressing the organisms surviving never reaches zero, although approaching it infinitely close. Mathematically speaking, then, sterility means that in the volume of liquid tested the number of surviving bacteria present is less than one.

This would seem to explain why it may take longer to sterilize a concentrated culture than one more dilute.

In summary, the effect of environment upon bacteria may be observed by any variation in the rate of growth, the rate of death, or the physiological or morphological state of the cell.

The physical agents affecting bacterial growth are temperature, light, electricity, moisture, osmotic pressure, and certain mechanical agents such as pressure and agitation.

Temperature relations. Temperature is an important factor influencing the growth of microorganisms. One of the first operations which the student ordinarily performs in the laboratory is the incubation of bacteriological cultures at temperatures suitable for growth. Some organisms possess marked ability to adapt themselves to temperature conditions. *B. subtilis* (the hay bacillus) grows through a wide range (6°–50° C.), while a pathogen such as *Mycobacterium tuberculosis*, which has become accustomed to the temperature of the body, has a narrow range (29°–42° C.).

Every organism, then, has a growth-temperature range; beyond certain limits growth will fall off and finally cease. There are three critical growth temperatures: the optimum temperature, or that at which the organism grows best; the minimum temperature, the lowest temperature at which growth still occurs; and the maximum temperature, the highest temperature at which growth and multiplication can take place. The following table will show how these three temperatures vary with different species:

TABLE V

SPECIES	TEMPERATURE IN CENTIGRADE		
	Minimum	Optimum	Maximum
<i>Bact. phosphoreum</i>	0	18	28
<i>B. subtilis</i>	6	30	50
<i>B. anthracis</i>	14	37	45
<i>Bact. thermophilus</i>	42	63–70	72
<i>Bact. ludwigii</i>	50	55–57	80

Bacteria have been conveniently divided into four groups according to their optimum temperatures. *Thermophiles* are those which develop at extraordinarily high temperatures, the

maximum being around 80° C., well above the temperature which ordinarily coagulates albumin. Such organisms are found in hot springs and in decaying piles of organic matter which develops considerable heat by fermentation. They are also sometimes present in milk, and may actually develop in milk during pasteurization, or in pasteurizing equipment.

Psychrophiles are organisms which develop at relatively low temperatures, even at or very near the freezing point. Such

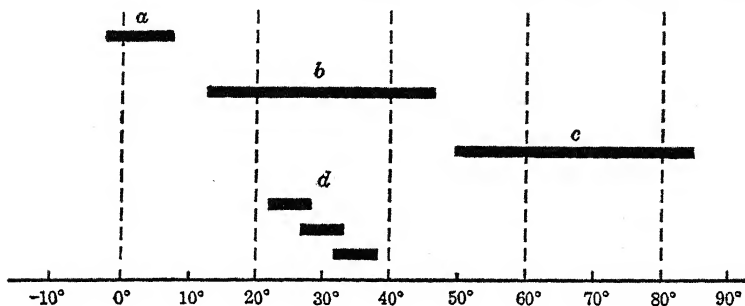


FIG. 33. Growth-temperature range of different groups of bacteria

a, psychrophiles; b, mesophiles; c, thermophiles; d, microphiles

bacteria are found in deep wells and lakes, and in the ocean. Some of them cause the spoilage of food in cold storage.

Mesophiles are those of the intermediate group, to which most organisms belong. This group may be subdivided into organisms which have as their optimum temperature 37° C. (body temperature) and those growing best below this, usually between 20° and 30° C. Yeasts have optimum temperatures between 20° and 30° C.; molds, slightly lower.

We have given the name *microphile* to organisms very limited in the temperature range in which growth is possible; minimum and maximum temperatures are separated by only a few degrees. Many of the pathogenic bacteria belong here, and grow at temperatures near that of the body of the host. Bacteria long accustomed to an environment of rather constant temperature will grow only within a relatively narrow range.

If bacteria are held at a temperature above their maximum, they will begin to die. Death may be accelerated by increasing

the temperature. A lower temperature for a longer period of time and a higher temperature for a shorter interval are equally efficient in bringing about sterilization. It will take longer to kill all the bacteria in a dense culture than in one less concentrated. The temperature at which complete sterilization of a twenty-four-hour-old pure culture occurs when exposed under standard conditions for ten minutes is known as the *Thermal Death Point*. Some of the factors which affect the thermal death point are moisture, reaction of the medium, nature of the organism, and resistance of spores.

The moisture present influences the thermal death point, moist heat being more destructive than dry heat. In other words, dried organisms are more resistant to high temperatures than are those in a liquid medium. It has been shown that the thermal death point of *Saccharomyces pasteurianus* is 105° C. for cells in the dried state, and 55° when moist.¹ Destruction is doubtless due to the coagulation of albuminous matter in protoplasm, which is facilitated by the presence of water and varies according to the percentage of water present. This is illustrated in the following table:²

Egg albumin + 50 per cent water coagulates at	56° C.
Egg albumin + 25 per cent water coagulates at	74°-80° C.
Egg albumin + 18 per cent water coagulates at	80°-90° C.
Egg albumin + 6 per cent water coagulates at	145° C.
Egg albumin + 0 per cent water coagulates at	160°-170° C.

The *reaction of the medium* exerts a marked influence on the thermal death point. The housewife knows that it is easier to can an acid food, such as tomatoes, than one less acid, such as corn or squash. Increased concentration of hydrogen ions makes the heat more effective. The graph on page 97 shows that *Esch. coli* multiplies in distilled water at pH 6.0 and pH 8.0, and in Ringer's solution at pH 6.0; but a strongly acid solution (pH 2.0) causes a rapid death of the cells, resulting in complete disinfection after four hours.³

¹ C. E. Marshall, Microbiology.

² Frost and McCampbell, General Bacteriology.

³ Shaughnessy and Criswell, "Studies on Salt Action," in *Journal of General Physiology*, Vol. IX, No. 2 (November 20, 1925), p. 131.

Another factor which influences the thermal death point is the species of the organism. There are differences between organisms, due to some inherent property possessed by the cell. One organism will be destroyed at a temperature of 60° C. applied for ten minutes, while another requires a higher temperature for sterilization. The application of heat in the pasteurization of milk is based upon an understanding of

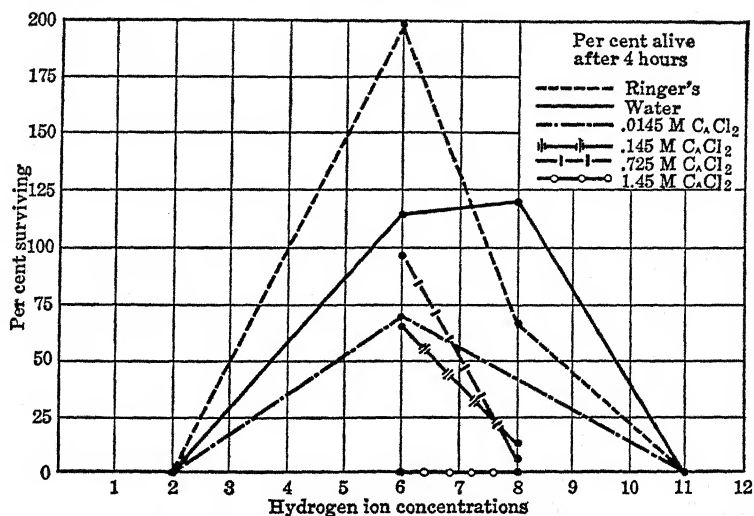


FIG. 34. The effect of varying hydrogen ion concentrations on the survival of *Esch. coli*

individual differences of organisms in their resistance to heat (see Fig. 35). The temperature used in the process is sufficient to kill the tubercle bacillus, while some of the putrefactive and lactic acid bacteria escape.

The presence of spores will profoundly influence the resistance of an organism to heat. When we are working with a culture of a spore-forming organism, we must take into account the fact that there is a temperature at which the vegetative cell is killed, and another, and much higher one, for the spore. Spores are extraordinarily resistant to heat; boiling does not readily kill them. Spores have been known to with-

stand the temperature of boiling water for sixteen hours. If the medium is heated to boiling temperature and held there for about fifteen minutes, all microorganisms are killed except certain spore-forming organisms. These spores germinate and require a second and third heating to complete the sterilization. Ordinarily heat is applied on three successive days for fifteen minutes. The time elapsing between successive heatings

is sufficient to allow for the germination of spores, which will then be killed at the next heating.

Spore-forming organisms are of economic importance in that they are responsible for so much spoilage of canned goods in the home.

Bacteria are much less sensitive to low than to high temperatures. Life processes are retarded by cold, but they do not cease entirely. It is for this reason that stock cul-

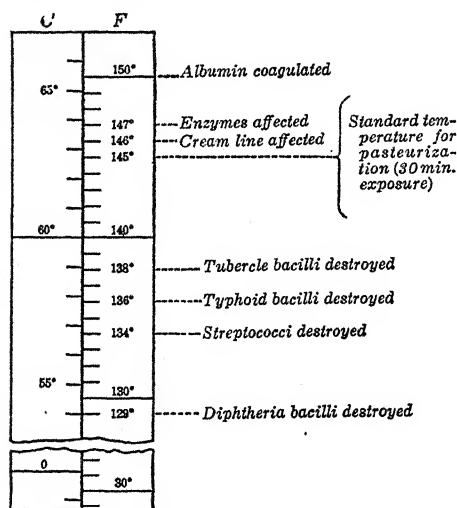


FIG. 35. The temperatures at which certain bacteria are destroyed during the pasteurization of milk for thirty minutes

tures are put in the ice box in order to preserve them.

The anthrax bacillus, whose optimum temperature is 37° C. and whose normal minimum temperature is 14° C., may be adapted to grow at a temperature of 10° C. by gradually decreasing the temperature so that the organism can adjust itself to this condition. A freezing temperature does not instantly destroy all bacteria; the death rate is, however, high. Ice is by no means a sterile product, as is commonly thought. Epidemics of typhoid fever have been attributed to the use of infected ice.

It has been noted that temperatures above the maximum for growth are injurious to bacteria. The death rate proceeds at a predictable rate, being accelerated as the temperature increases.

Temperatures below the minimum, on the contrary, exert a protective influence. Metabolism is arrested to its lowest ebb, and with most bacteria, excepting the psychrophiles, reproduction ceases completely below $6^{\circ}\text{C}.$, and the bacteria die gradually, some surviving for weeks. The typhoid bacillus has been shown to survive in ice for as long as twelve weeks. Cold, as such, evidently acts as a preserver of bacterial life rather than as a destroyer. Living cultures keep best in the ice box. In a dry state bacteria will survive exposure to the temperature of liquid air ($-190^{\circ}\text{C}.$) or liquid hydrogen ($-252^{\circ}\text{C}.$).

It is evident that a low temperature cannot be relied upon for sanitary purposes or for sterilization. The presence of suspended matter, as particles of sewage in water, will provide protection for bacteria even when frozen solid. Yet all pathogenic bacteria die gradually at refrigeration temperatures, and so in this respect foods that have had a period of storage are safer than are fresh products.

When we consider temperatures that actually freeze, or crystallize, water, the situation is different. Freezing exerts a powerful and apparently an instantaneous lethal action. In pure water inoculated with a culture, frozen, and then thawed and tested immediately, more than 90 per cent of the bacteria are destroyed. If a similar preparation is alternately frozen and thawed, the culture may be nearly sterilized in a short time. If the tube of water is shaken to inject air bubbles, making it of sherbet consistency instead of solid ice, the death rate is much lower; or if we substitute milk or cream or any medium with colloidal or suspended matter present, fewer bacteria are destroyed. These facts are brought out by the table on the following page, taken from a study made by the author.¹

¹ C. M. Hilliard and Mildred A. Davis, in *Journal of Bacteriology*, Vol. III, No. 4, p. 426.

TABLE VI. PERCENTAGE REDUCTION OF BACT. COLI SUSPENDED IN WATER AND CREAM AND FROZEN AT -15° C.

	FIRST FREEZING PERCENTAGE REDUCTION	SECOND FREEZING PERCENTAGE REDUCTION	THIRD FREEZING PERCENTAGE REDUCTION	FOURTH FREEZING PERCENTAGE REDUCTION
Water	92.8	96.1	99.8	99.9
	97.6	99.6	99.5	99.9
	98.6	99.4	99.8	99.8
Cream	32.2	36.2	48.4	71.7
	43.1	46.7	71.9	81.2
	40.5	45.5	71.5	75.9

Further experiments showed that when the freezing temperature was depressed by the presence of a harmless solute in water, the death rate was very much lower — about one half — than when water was frozen at the same temperature.

It is evident that the crystallization of the water and the composition of the medium have much to do with the destructive action exhibited at low temperatures. It is probably due to the pressure, and to grinding developed between the crystals, resulting in the mechanical crushing of the cells, rather than to the germicidal action of cold. Fat globules, air bubbles, or any suspended matter giving a refuge to the bacteria from this action will lower the death rate.

It should perhaps be emphasized again that low temperatures retard or completely prevent the multiplication of bacteria, and that the home ice box and the commercial refrigeration of food are based upon that fact.

The influence of light. The bacteria, being colorless plants, do not utilize the radiant energy of the sun in building up their food material; on the contrary, light is destructive to them. This may be seen by a simple laboratory experiment. If we inoculate an agar plate with the organism to be tested, paste a piece of black paper in the form of some letter on the under side, expose the inverted plate to the direct rays of the sun for an hour or more, and then incubate, it will be found that the growth, when examined after incubation, takes the form

of the figure used, while the part exposed to the sunlight shows little or no growth to have occurred.

The destructive action of sunlight is not due to heat rays, as might be supposed. When the spectrum is broken up into its component parts, we find that the ultra-violet rays are the most germicidal. Glass absorbs the destructive rays to an appreciable extent, thus diminishing the bactericidal action.

The effect which is brought about by exposure to light is due probably to photochemical changes in the protoplasm, and perhaps also to the formation of some oxidizing agent in the suspending medium. Light produces no effect on bacteria in a vacuum.

Sunlight is universal, and its relation to hygienic living cannot be overemphasized. Bacteria cannot long survive

when exposed to the direct rays of the sun, either in a sun-filled room or on the surface of a water supply. Many factors must be taken into consideration, as is the case in dealing with any lethal agent — the time of exposure, the intensity of the light, and the nature of the organism. Some microorganisms are stimulated by light and grow toward it. Molds show this property of phototropism. Some spores are apparently not affected by sunlight, while others are quickly killed.



FIG. 36. Petri dish showing the disinfecting action of the sun on *Esch. coli*

The surface of the medium was uniformly seeded, and the portion indicated by the growth in the shape of the cross was protected by heavy, dark paper. The medium was exposed to the direct rays of the sun for about one and one-half hours. Practically all the bacteria in the exposed portions were killed

The electric light is not nearly so harmful to microörganisms as is direct sunlight. The use of ultra-violet light in curing diseases of the skin and scalp has become an important therapy. The Röntgen ray, or X-ray, and radium emanations have germicidal properties. These rays are destructive to the body cells as well as to the bacteria; so their application is limited.

The influence of electricity. Passing a direct electric current through a bacterial culture apparently has no destructive action. Harmful substances, however, may be liberated by electrolytic dissociation. This has been taken advantage of in the purification of sewage effluent by the electric current, and in the purification of water supplies by the liberation of ozone. Bacteria, being negatively charged, are carried to the positive pole when a weak electric current is passed through the suspending medium. An alternating current may heat a medium to a temperature that will be lethal. This is used to some extent now to pasteurize milk.

Influence of moisture. Nature has given us a method for the preservation of the seeds of the higher plants by desiccation, or drying. We plant these dry, dead-looking seeds in the ground in the early spring and keep them well watered, and we soon see the young plants developing. Desiccation is likewise a means of preserving bacteria. In a dried state they remain inactive for some time, and may later be revived and cultivated. Great resistance to drying is shown by some bacteria; the spores are much more resistant than the vegetative cells.

We have spoken of the necessity of *moisture* for bacterial growth. Diffusion of food products occurs between the cell and the outside medium through the agency of water. Deprived of this the cell can no longer function. When bacteria are dried or frozen in a medium they are deprived of the water necessary for the diffusion of foods in solution; the physiological activities of the cells then come to a standstill. Drying has long been used as a method of retarding bacterial growth to preserve food. Meats, vegetables, and fruits are sun-cured or dried by artificial means.

Osmotic pressure. In the chapter on the physiology of bacteria attention was called to the fact that foods in solution pass through the semipermeable membrane of the cell, and excretions leave the cell through this membrane. If we put some salt into a glass of water, the molecules are dispersed throughout the water until a solution of a definite concentration results. Salt or sugar added to the medium in which bacteria are growing will increase the concentration, thus causing an increased osmotic pressure. The pressure within the cell is lower than that existing in the medium; water will therefore leave the cell until the pressure on both sides is equalized. This loss of water causes a shrinking of the protoplasm, drawing the cell membrane in away from the rigid cell wall. This phenomenon is known as *plasmolysis* (see Fig. 30). The antithesis of this is *plasmoptysis*, which occurs when the cell is immersed in a hypotonic solution, or one of a lower concentration than that which exists within the cell. Water then enters the cell until the pressures become equal. This causes a swelling, and if continued the cell will finally burst.

The effect of strong salt and sugar solutions in pickling and canning is one of plasmolysis. The chemical nature of the salt plays some part as well. The growth of most microorganisms is suppressed by a 10 per cent concentration of salt; the torulæ, however, are found in pickles, salt pork, and butter in concentrations above 20 per cent. This resistance offered by some organisms to high salt solutions is probably due to the fact that the salts, as well as the water, diffuse through, keeping the pressures equal on both sides of the membrane. The difference exhibited by bacteria in their resistance to plasmolysis is taken advantage of in the manufacture of some food products such as sauerkraut, where the desirable fermentation is brought about by organisms able to withstand the salt concentration, while undesirable fermentations are prevented.

Certain mechanical agents, such as pressure and agitation, are known to affect bacterial growth. A quiet medium is one most favorable to growth. Gently swinging or agitating a broth culture apparently has little effect. Prolonged, violent

shaking is harmful, owing to the breaking apart of the cells. This effect is increased when bacteria are shaken with glass beads, in which case some are mechanically crushed.

Bacteria are found alive in the ooze at the bottom of the ocean, proving that they have great resistance to changes in pressure. Marshall states that "pressures of 3000 atmospheres will not kill the majority of bacteria in four hours." We have an entirely different situation if the bacteria are exposed to compressed air; the increased pressure of the oxygen gas is then the lethal agent. "Carbon dioxide at a pressure of 50 atmospheres retards the growth of bacteria in water and will sterilize it in twenty-four hours."

Physical influences of bacteria upon their environment. We have considered the various ways in which the physical environment affects the growth of bacteria as if they played a merely passive rôle. Let us now turn to a consideration of certain physical phenomena brought about by bacterial growth — heat and light production, pigment production, changes in the consistency of a medium, and clouding or turbidity.

A breaking-down process means a release of energy; when all the energy is not utilized by the bacteria causing the fermentation for their vital processes, some escapes in the form of heat. Ordinarily fermentation changes are accompanied by a very slight rise in temperature. It is only when there is a marked rise in temperature that we speak of these heat-liberating organisms as *thermogenic*. Temperatures as high as 70° C. may be reached. In the commercial manufacture of vinegar, heat production is so great that the process is carefully controlled. Perhaps the most familiar example is the production of heat in the fermentation of manure and ensilage. Just what part bacteria play in the heat produced in silos is not fully understood. It is sometimes claimed that the heat evolved in haylofts due to fermentation is sufficient to bring about spontaneous combustion. This is a fallacy, as the high temperature necessary to cause the hay actually to burst into flame would be destructive to bacteria and their enzymes. It has been shown that sterile hay will show a rise in tempera-

ture. The latest observations support the theory that the self-heating of hay, tobacco, and moist grains is of a purely chemical nature and is due to changes brought about by the plant cells.

A practical use of heat production due to bacterial growth is seen in hotbeds for the cultivation and forcing of young plants.

The light-producing, or *photogenic*, bacteria are of great interest to the student. The phosphorescence of sea water and decaying fish along the shore is due to these organisms. The peculiar "fox fire" seen on decaying wood and leaves in the forest is commonly caused by these light-producing organisms, although sometimes by the molds.

The phenomenon is apparently the result of an oxidation process, as oxygen is necessary for photogenesis. Light is produced within the cell, owing to a substance known as photogen. Certain chemicals, such as sodium and magnesium, are known to stimulate the production of light. If a watch is held near the culture in a dark closet, one can tell the time by the light produced; cultures of bacteria have even been photographed by their own light.

Pigment production, or *chromogenesis*, is another striking result of bacterial activity. The range of colors seen is considerable; red and yellow pigment, however, are the most common.

Red	<i>Serratia marcescens</i>
Orange	<i>Sarcina aurantiaca</i>
Yellow	<i>Staph. citreus</i> , <i>Sarcina lutea</i>
Green	<i>Pseudomonas æruginosa</i>
Blue	<i>Pseudomonas cyanogena</i>
Violet	<i>Chromobacterium violaceum</i>

The purpose of pigment production is still unknown. *Ser. marcescens* grows quite as well at 37° C., with diminished pigment production, as it does at 20° C. Pigments do not appear to take the place of the chlorophyll of higher plants except perhaps in the case of *bacteriopurpurin*. The red pigment produced by some bacteria appears to have an affinity for oxygen, analogous to that of hæmoglobin. In most cases, however, the pigments are considered merely as by-products, their forma-

tion being incidental and not essential to metabolism. Pigment production does not protect bacteria against light, as is the case with certain mold spores.

Pigments may remain in the cell in the form of granules or may be excreted from the cell. They may be classified as to their solubility in water, ether, chloroform, and various fat solvents. If soluble in water, they will of course diffuse throughout the medium. Little is known of their chemical nature; the majority are related to the lipochromes, or fatty pigments. Certain mineral salts, definite temperatures, and aërobic conditions usually stimulate pigment production. *Spirillum rubrum* is one of very few organisms which produce pigment anaërobically.

The consistency of the medium may be changed as the result of bacterial growth. Milk inoculated with a culture of *B. subtilis* is first changed to an insoluble curd and later digested, resulting in a thin, amber-colored liquid. We could give many examples of analytic processes: starch paste inoculated with an amylolytic organism is changed to a thin liquid; cellulose is hydrolyzed into the simple sugars by some bacteria; a solid gelatin stab culture becomes liquefied after inoculation with some proteolytic organisms.

Some organisms may synthesize products, such as slimes, gums, and mucin-like substances, which change the viscosity of the medium. The Type III pneumococcus has a heavy, polysaccharide capsule which gives to the exudate a slimy character. Certain gumlike substances are synthesized by bacteria.

Any change in a medium which results in an increased number of molecules increases the osmotic pressure. Analytic processes will then increase osmotic pressure, while synthetic changes will decrease the pressure.

The porosity or texture of a medium may be altered by the growth of microorganisms, as in the production of bread and cheese.

Lastly, a broth culture may be uniformly cloudy, or turbid, or may show the formation of a precipitate, as the result of bacterial growth. The bacteriologist makes use of this as an index of growth.

CHAPTER IX

BACTERIA AND THE CHEMICAL ENVIRONMENT

The metabolism of bacteria, and the substances that serve as food, have been discussed, so that this chapter will be devoted to the injurious and destructive action of chemical agents.

The chemical substances are employed for antiseptics or disinfection. *Antiseptics* are substances that inhibit or suppress growth but do not necessarily destroy microbes. When they exert their action over a sufficient period of time, a slow elimination of the bacteria present may result. *Disinfectants* are agents that actually kill bacteria in a short time — in seconds or in a few minutes. The line of distinction between disinfectants and antiseptics is not sharp and clear-cut, for a disinfectant for one organism may act as an antiseptic for another, at the same concentration, or the same substance diluted beyond a certain concentration may exert a mild antiseptic action only. The selective action of certain chemicals is strikingly shown in the case of certain dye substances. Gentian violet and other dyes of the triphenylmethane group exert a powerful disinfecting (bacteriostatic) action toward most of the Gram-positive organisms but do not affect the Gram-negative forms at all. The difference between the concentrations of the same substance required to inhibit and to destroy an organism is illustrated by the fact that anthrax spores will not develop in a 1:300,000 dilution of mercuric chloride, while to kill them it requires a 1:1000 dilution.

The disinfectant to be used in any given instance must be determined by the particular object to be attained. Poisonous chemicals cannot be added to water and food, or used for gargles or internal disinfection. Corrosive acids or metallic salts cannot be applied to the skin. Too expensive reagents are not justified for general uses in the sick room or about the

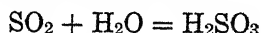
home. Many proprietaries are unduly expensive, and claims for them are often unwarranted. Great numbers of these are on the market, but as a general rule it is better to select a few well-known and simple chemicals than to experiment with the newer, little-known compounds about which we lack reliable information.

In a given case the following are the principal factors that will determine the disinfecting action of a chemical: (1) the concentration of the reagent; (2) the time over which it is allowed to act; (3) the presence of organic matter in the material to be treated; (4) the presence of free moisture; (5) the organisms to be killed; (6) with metallic salts, the degree of dissociation that occurs; (7) the pH concentration.

The views as to the importance and application of disinfection have undergone a change in the last twenty-five years. In the days when the belief in the general environmental origin of certain disease germs was prevalent, and bad odors were confused with infectious material, it seemed as though it were important to disinfect and deodorize promiscuously. The air-borne theory of infection focused attention upon the atmosphere and objects generally rather than upon specific infectious matter emanating from the sick patient or from other persons. Fumigation after sickness was a feeble and unreliable effort to disinfect the air and objects in the sick room. The confidence placed in disinfection often led to carelessness in regard to scrupulous cleanliness. Today little reliance is placed in fumigation, and this expensive and useless practice has been generally abandoned. It has a limited value today in destroying insects and other vermin, and possibly, in exceptional cases, following disease. We should no longer confuse deodorization with disinfection. It is fitting to substitute "clean" odors in public toilets, but this is not a substitute for cleanliness. The application of chemicals to the environment is an admission that contamination or infection has occurred, and it is at best a poor substitute for sanitation in its broadest sense. The liberal use of hot water, soap, and "elbow grease" will make disinfection unnecessary in most cases.

Disinfection has specific rather than general applications. The right disinfectant should be applied in the right way at the right time and place. In surgery, and as concurrently used during the course of an infectious disease, it is of greatest importance. The discovery and use of internal disinfectants (chemotherapy) has a promising future. The use of antiseptics in food and the disinfection of water will receive attention elsewhere.

Fumigation. Fumigation is disinfection of cubic spaces by the use of volatile or gaseous agents. The burning of *sulphur* is the classic method. Sulphur dioxide (SO_2) is formed and in the presence of abundant moisture forms sulphurous acid, which is the active germicidal agent.



From four to five pounds of sulphur are required for each thousand cubic feet of space, and water (roughly, one-fifth pound to each pound of sulphur) must be artificially volatilized. Insects and rats are killed by sulphur fumes, which is an advantage of this method over formaldehyde fumigation. On the other hand, the fumes are poisonous to man, and they are injurious to fabrics and metals.

Formaldehyde fumes (HCHO) are formed in various ways — by the evaporation of the 33–40 per cent solution known as formalin; by the rapid volatilization of this solution by pouring it over potassium permanganate crystals; or by the heating of the solid polymer of formaldehyde (paraform), which may be purchased in convenient form, together with a special lamp for heating. Twelve ounces of formalin per thousand cubic feet is usually recommended. Paraform has its special directions for use accompanying each purchase. The gas is active only when in solution, and the air must therefore be moist. Formaldehyde is more penetrating than sulphur fumes and does not injure fabrics or metals. On the other hand, insects and vermin are not destroyed.

Hydrocyanic acid gas is used especially in the fumigation of ships. It is a powerful poison and does not lend itself to household uses.

Disinfection of the skin and of infectious matter. The liberal use of *soap*, warm water, and scrubbing will accomplish much in the removal of bacteria as well as dirt. The fatty acids, formed by the splitting of the alkali salts in the soap, have a disinfecting action also. The disinfecting action of soaps has a specially important practical bearing, as it is so universally used in hand-washing, dish-washing, and laundering.

It has been shown that different soaps vary greatly in their germicidal action, and that bacteria vary greatly in their resistance. Pneumococci, for example, are killed in fifteen minutes by an $N/10,240$ (approximately 1:50,000) solution of sodium laurate, while the ubiquitous staphylococcus is almost completely resistant to the most concentrated solutions of this soap.¹ The typhoid bacillus is resistant to most soaps, but the coconut-oil soaps have been shown to have a selective germicidal action for this organism.² Dissolved in 2 per cent NaCl in water, a coconut-oil soap destroyed typhoid in 1:1000 dilution, while linseed-oil soaps prepared in a similar way required 1:30 dilution to be effective.

The following table is interesting as showing the effect of commercial soaps on different pathogenic bacteria:

TABLE VII. EXPERIMENTS SHOWING THE KILLING STRENGTH OF VARIOUS SOAPS³

	B. DIPHTHERIÆ	STREP. HÆMOLYTICUS		B. TYPHOSUS			STAPH. AUREUS	
	20° C.	20° C.		20° C.		35° C.	20° C.	35° C.
	15 min.	2½ min.	15 min.	2½ min.	15 min.	15 min.	15 min.	15 min.
White floating .	1:1280	1:320	1:640	+	+	1:160	+	—
Perfumed toilet	1:2560	1:160	1:640	+	+	1:160	+	—
Sapo mollis . .	1:1280	1:160	1:320	—	—	1:20	—	—
Brown bar . . .	1:1280	1:80	1:160	+	+	1:160	+	1:10
Phenol	1%	2%	1%	2%	1%	½%	2%	1%

The minus sign indicates that the strongest solution prepared (1:10 for all soaps except *sapo mollis*, in which it was 1:25) did not kill the organisms.

These soaps form gels at 20° C. in dilution stronger than 1:160.

¹ J. E. Walker, in *Journal of Infectious Diseases*, Vol. XXXV, p. 566.

² Tilley and Schaffer, in *Journal of Infectious Diseases*, Vol. XXXVII, p. 362.

³ J. E. Walker, in *Journal of Infectious Diseases*, Vol. XXXV, p. 183.

The percentage of soap in the lather present when the hands are thoroughly washed has been found to average roughly 8 per cent. With prolonged washing and soaping it may rise to as high as 20 per cent (Walker). It seems as though thorough soaping, and contact with the lather for some minutes, especially accompanied by scrubbing to effect loosening and mixing of the bacteria with the lather, will accomplish much in the way of bacterial removal and destruction. In dish-washing the temperature of the water is an added destructive factor.

Washing with soap cannot be relied on to remove all bacteria or to disinfect, and in the preparation of the skin for surgery or in the treatment of minor cuts and scratches, or of wounds, this method cannot be used. The search for the ideal disinfectant for the treatment of wounds is fraught with great difficulties. An agent that does not injure the tissues, that penetrates, and that will combine with bacteria in the presence of albuminous matter in the blood and tissues must be found. Tincture of *iodine*, usually as a $2\frac{1}{2}$ per cent solution in pure 70 per cent alcohol, is an old stand-by for treating minor cuts, and in some instances in preparing the field of operation. A compound of iodine, *iodoform* (CHI_3), has been much used as an antiseptic in dry dressings. *Alcohol*, though expensive, is a fairly satisfactory surface disinfectant. It is most active in a 50-70 per cent dilution, as sufficient water is essential for its action. Certain dyes, as gentian violet, brilliant green, or acriflavine, have special uses as skin disinfectants.

Mercuric chloride (HgCl_2) is one of the best-known and most effective of the metallic salts. For disinfecting the hands or intact skin surfaces it is effective in a solution of 1:1000. A trace of acid or a small quantity of common salt (NaCl) will enhance the action of this substance by increasing the degree of ionization. In the presence of organic matter, as blood or pus, the mercury ions enter into inert combination and render it ineffective. This mercury salt is a powerful internal poison, and it also corrodes metals. Other metallic salts extensively used for disinfection are copper sulphate, ferrous sulphate,

and zinc chloride. The first has special value as an algicide, to rid water supplies of certain microscopic green plants that cause offensive tastes and odors.

Perhaps the best-known and most universal of all disinfectants are those derived from coal-tar products, of which *carbolic acid*, or phenol (C_6H_5OH), is an example. Lysol, cresol, tricresol, and other closely related organic substances are more powerful germicides than phenol. Five per cent carbolic is a fairly reliable agent, killing all vegetative and spore-bearing bacteria in a few minutes. It is active even in the presence of organic matter. For this reason it is of special value in the disinfection of sputum from tuberculous or other patients, and the bowel and bladder discharges from typhoid or other intestinal diseases.

Calcium compounds, especially calcium hypochlorite, or Bleaching Powder, deserve special mention. Calcium hypochlorite is cheap and, freshly made up in 5 per cent solution, is a powerful, reliable germicide. As a dry powder it is ineffective, and when sprinkled in privy vaults in this form it merely deodorizes. If thoroughly mixed with the contents of the bedpan and allowed to stand for half an hour at least, it will kill any disease bacteria present. Hypochlorite was originally extensively used in water purification, but it has now been largely supplanted by liquid chlorine.

The list of available disinfectants is very extensive, and enumerating a large number of them does not serve the purpose of this text. With the extension of the field of chemistry new and better compounds will be discovered. Of special interest at this time are mercurochrome and various dye substances, including gentian violet, acriviolet, and brilliant green. *Mercurochrome*, which contains from 23 to 26 per cent of mercury, holds special interest because of its powerful selective germicidal action in the presence of organic matter. It is a better skin disinfectant than either iodine or alcohol; but, more important, it may be injected in appreciable doses intravenously, and while the results are as yet far from conclusive, there is some hope that it or a related substance may

soon be discovered which may be safely used for internal disinfection in septicæmias and other diseases. The dye substances are also effective as local antiseptics on the skin, in open wounds, and in joint infections. The study of this group of reagents seems sure to be on the verge of some brilliant and important discoveries. The path of specific chemotherapy has been blazed in the case of the blood infections caused by the Spirochætes. The famous "606," or salvarsan (arsphenamine), a compound containing combined arsenic, was discovered by Ehrlich in 1910 as a cure for syphilis, which is caused by the *Treponema pallidum*.

The testing of disinfectants. With such a diversity of disinfectants on the market it is important that some standard be set, so that the relative germicidal value of different reagents may be tested. Extravagant claims made in advertisements should be checked up so that the purchaser may have some idea of the value of the products which he buys and of their cost in terms of their disinfecting properties. The layman himself has no means of determining whether or not materials are sterilized or whether a particular agent should be diluted ten or one thousand times when used.

There are two general tests that may be applied to demonstrate the activity of chemicals toward germ life. One is the test of antiseptic action and the other the test of disinfecting action.

The *inhibition coefficient* may be considered as the minimum concentration of a given chemical substance that will completely prevent the growth of an organism. A series of dilutions of the chemical to be tested are made, using either bouillon or liquefied agar as the diluting medium, and, after thorough mixing, each tube is inoculated with a loopful of the culture. The tubes are incubated, and examined after forty-eight hours for growth. The tube of lowest concentration in which no growth has occurred represents the inhibiting strength of the chemical. This test has a limited use and has received no official sanction. It has value, however, in determining the smallest quantities of a chemical that may be

added to food or to other products containing organic substances in order to assure their preservation. The legitimate application of chemical preservatives to foods is very limited.

The *phenol coefficient* is an arithmetical expression of the disinfecting strength of the chemical being tested, in terms of the strength of pure phenol. The determination of this coefficient should be made by a uniform, standard procedure, so that one proprietary may be compared with another. The official test of disinfectants in this country is performed by the method described by the Food and Drug Administration, United States Department of Agriculture,¹ although in some laboratories the so-called Rideal-Walker method is used. The principles involved in the two tests are similar.

The *test culture* is a known strain of *Eberthella typhi*, which is grown and transferred in the laboratory under prescribed conditions. The test is always performed with a twenty-two-hour to twenty-six-hour culture.

Pure phenol crystals are used to make up a 5 per cent original dilution used in the test.

Carefully calibrated pipettes and cylinders are used to make up the dilutions of the phenol and the chemical to be tested. The phenol is ordinarily made up in dilutions 1:90 and 1:100, and 5 cc. of each dilution is placed in a test tube. The unknown chemical may first be roughly tested to get the range of its disinfecting properties. Appropriate dilutions are then made up for the test, and 5 cc. of each dilution is placed in test tubes. Then $\frac{5}{10}$ cc. of the approximately twenty-four-hour-old stock culture is run into each tube at 30-second intervals.

At the end of 5 minutes from the time of adding the disinfectant to the first seeding tube a loopful of the mixture is transferred from this tube to a subculture tube, and this is done from each successive seeding tube at 30-second intervals. This procedure is repeated after the lapse of 10 and 15 minutes from the time of the first addition of culture to the seeding tube.

¹ United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants. *Circular No. 198* (1931), United States Department of Agriculture.

After forty-eight hours' incubation at 37° C. the tubes are removed and studied for the presence of growth and are entered in a table as + (growth) or - (no growth).

The results of the test are expressed in terms of the phenol coefficient. This represents the germicidal value of the diluted disinfectant as compared with the diluted phenol control. It is a figure obtained by dividing the numerical value of the greatest dilution (the denominator of the fraction expressing the dilution) of the disinfectant capable of killing *Eberthella typhi* in 10 minutes but not in 5 minutes, by the greatest dilution of phenol showing the same results; that is, by the phenol control. Thus, if the results were as in the following table, the phenol coefficient would be $\frac{350}{90} = 3.89$.

TABLE VIII¹

SAMPLE	DILUTION	TIME OF EXPOSURE		
		5 min.	10 min.	15 min.
Unknown disinfectant	1-300	-	-	-
	1-325	+	-	-
	1-350	+	-	-
	1-375	+	+	-
	1-400	+	+	+
Phenol	1-90	+	-	-
	1-100	+	+	+

In the example given the coefficient 3.89 means that under test conditions the unknown chemical was about four times as powerful a germicide as 5 per cent pure phenol. Had the coefficient been less than 1.0, the strength of the agent being tested would be less than that of 5 per cent pure phenol. A reliable disinfectant should state clearly on the label its phenol coefficient.

As has been warned already, for given purposes there may be other considerations than germicidal power. On the other hand, an expensive though feeble disinfectant has few or no reliable uses, while a cheaper and strong one could be relied upon for many purposes.

¹ United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants. Circular No. 198 (1931), United States Department of Agriculture.

CHAPTER X

ENZYMES. THE FERMENTATION OF CARBON COMPOUNDS

The part played by enzymes in cell nutrition is of supreme importance. This is true not only of bacteria and other microorganisms but of all higher organisms, including man. It will be necessary, therefore, to devote some detailed consideration to the nature and properties of these extraordinary substances that we call enzymes.

In the early days enzymes (or, as they were then called, ferments) and microorganisms were confused. It was supposed that fermentation in sugar solutions, for example, was purely a chemical process, and that yeast cells either were a product of fermentation or were incidental to the fermenting activity. Next it was proved that certain fermentations might take place in the presence of the cell-free filtrates of yeast cultures. These were referred to as unorganized ferments, while the changes carried on only in the presence of the living cells were said to be caused by organized ferments. We now recognize a distinction in these two cases, but do not consider that the nature of the agents involved is different; in the one case the enzyme is secreted by the cell and acts upon chemicals outside of the cell, while in the other case the enzymes are retained within the cell wall and ordinarily act only in this site. It has been possible, however, to crush cells and extract the latter enzymes, proving that they are not essentially different. We now refer to them as extracellular enzymes and intracellular enzymes (endoenzymes).

Enzymes may be simply characterized as organic or biochemical catalysts that are dispersed in water. Catalysts are agents which accelerate chemical reactions but which do not themselves enter into any new permanent chemical combina-

tions. It follows, therefore, that the amount of reaction is not proportionate to the amount of enzyme present. For example, finely divided platinum, called colloidal or spongy platinum, rapidly oxidizes the fumes of ethyl alcohol when the two are brought together in the presence of air, the alcohol finally bursting into flame. This is the principle of the familiar pocket cigar-lighter. The alcohol is used up in the reaction, but the catalyst, the platinum in a special physical state, continues to act indefinitely.

Enzymes are more than catalysts: they are organic catalysts; and we are concerned chiefly with their peculiarities rather than with catalysts as such. They are always the product of living cells. We have noted that they may act outside of the cell, ordinarily upon foods that are not diffusible into the cell, or that they may act within the cell upon diffusible substances. Therefore we find enzymes playing many important parts in the plant and animal world. The digestion of food in the alimentary canal of animals, the germination of seeds, and the ripening of fruit are common examples in our everyday life. All of them have properties in common.

1. Enzymes are colloidal in nature, giving the chemical reaction of proteins. They are not dialyzable.

2. Enzymes are specific in their action, each one causing a particular chemical change upon one substance. The number of enzymes must therefore be as numerous as the number of chemical substances that are acted upon. This law of specificity may be taken as a basis for classification, and commonly the name given to the particular enzyme is the name of the product acted upon with the suffix *ase*, as *maltase* or *lactase*, the enzymes splitting maltose and lactose respectively.

3. The chemical action brought about by enzymes never goes to completion, but there is always some of the mother substance being acted upon remaining in solution. This is explained by the fact that all enzyme reactions are reversible, and a point is always reached in the concentration of the new products where the reaction is taking place in opposite directions at the same rate.

4. Enzymes are sensitive to physical conditions of the environment. The rate of reaction is determined by the temperature. Most purely chemical reactions take place, for practical purposes, instantaneously. The rate of action of enzymes is measurable, and we note, as with bacteria, an optimum temperature, a minimum, and a maximum. They are inactivated by cooling and freezing temperatures, but are not destroyed. They are permanently destroyed by high temperatures. Usually, however, the destructive temperatures are higher than those which kill the cells producing the enzymes. They are inactivated by drying but are not destroyed.

5. Enzymes are sensitive to chemical conditions. Their activation may depend upon the presence of certain salts in solution or may be affected by the hydrogen ion concentration, some acting best in a slightly acid medium, others in an alkaline medium. They are precipitated by alcohol and by those salts that ordinarily precipitate organic colloids.

The enzymes are classified in various ways. They are grouped according to the nature of the chemical compounds acted upon. Those acting upon or splitting carbohydrate compounds are called *amylolytic*, those acting upon fats are *lipolytic*, and those acting upon proteins are called *proteolytic*. Another method of classifying enzymes is according to the nature of the chemical action that they induce. On this basis we speak of hydrolases (enzymes that split molecules by the addition of water), oxidases (enzymes that act by oxidation), reductases (those that reduce, or deprive chemicals of oxygen), and the zymases (those that split the chemical molecules directly without the addition or subtraction of any element or compound).

There are numerous practical applications of microenzymes (zymotechnique) to man's welfare. We have referred to the natural ripening of fruit, which may go on to overripening and spoilage. If an apple is bitten into and left for a few moments, it is familiar to find that the exposed surface is slowly turning brown. This is caused by an oxidase in the fruit tissue, which becomes active in contact with the air. The alterations that

occur in meat products during storage and the ripening process are due in part to autolytic enzymes, or enzymes in the tissues themselves, and of course are due partly to molds and bacteria that invade the tissues. The whole problem of food conservation is concerned with the suppression of the action of enzymes. The preparation of various food products, as bread, sauerkraut, butter, and cheese, involves fermentations carried on by enzymes

derived from various microbes. A group of extensive industries is based upon fermentations, as brewing and the manufacture of alcohol, acetic acid, and lactic acid. The fertility of the soil and the disposal of liquid wastes like sewage depend fundamentally upon the action of enzymes derived from bacteria. Even the phenomena of disease and immunity are

closely linked to the problem of enzymes, as we find that the microbial poisons and some of the immune substances have certain properties of enzymes, although they are not usually identical with these organic catalysts.

For practical purposes we may consider applied fermentations in two subdivisions: those that act upon carbon compounds and those that act upon nitrogen compounds. In its widest conception the problem involves the circulation of these elements in nature. It is no exaggeration that the continuation of life upon the earth is dependent upon continuing activities of various groups of microorganisms. The carbon and nitrogen elements that make up the living substance of

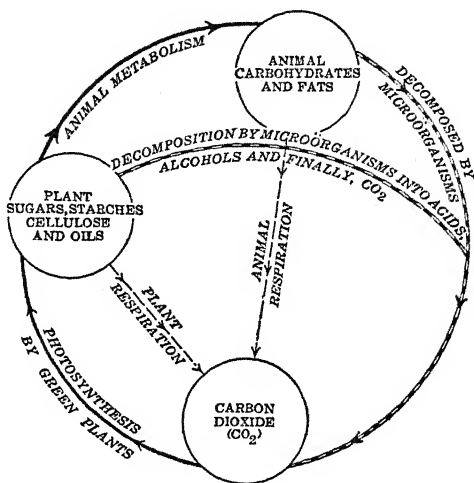


FIG. 37. The carbon cycle in nature, showing the rôle of microorganisms

our own bodies and of all other living things were at some time brought into the cycle of organic nature, and from the remotest time have successively passed through the bodies of other living animals or plants, sooner or later to be acted upon by microorganisms and broken into those simple elements which provide the green plant with food. The plant substance is in turn, either directly or indirectly, the sole source of human food.

We may take up first the circulation of carbon in the organic cycle. No carbon compounds are immune from attack by bacteria. The most resistant compound is cellulose, which makes up the woody fiber of plants; but we witness the slow disintegration of the tree trunk or stump into the mold and humus of the forest soil, due to the action of molds and bacteria.

Sugars and starches are the more familiar carbohydrate compounds. They are the principal substances that yield animals ready energy. In their breaking up, the chief end products that they yield are organic acids, the most prominent being lactic acid and acetic acid, ethyl alcohol, carbon dioxide, hydrogen, and water. It would be most helpful to illustrate in some detail the action of enzymes in one specific instance, and we shall select the common alcoholic fermentation as it is carried on in the brewing of beer. The process takes place in three steps.

Manufacture of beer. 1. *Malting.* The seeds of certain cereals, as barley, are soaked in water for two or three days. This stimulates the rapid germination of seeds, due to the activation of several enzymes, chiefly diastase, which hydrolyzes the starches in the seed to form the sugar, maltose. The process is watched, and when the sprout, or plumule, has developed to about two thirds of the length of the grain, the enzymes will have reached their maximum. This takes from sixteen to twenty days ordinarily. At this point the germination is arrested by the drying and heating of the seedling. The heat must be raised gradually, first from 30° to 55° C. and then more slowly, during the next twelve to twenty-four hours, to

80° and 100° C. This prevents the destruction of the enzymes, which would be permanently destroyed at the boiling temperature in the moist condition.

2. *Mashing.* The seeds are now crushed and warm water is added. Additional unmalted grain, as corn, may be added at this time, and the maltose and dextrins are produced. The temperature control is very important at this time, both to provide the best condition for the action of the diastase and also to prevent the action of acid-producing bacteria. When a maximum of maltose has been produced, this mash is boiled in order to sterilize it, and hops are added, which give the bitter taste. It is then strained and cooled rapidly to a temperature of from 6° to 12° C., according to the after-treatment to be administered.

3. *Fermentation.* The brewers' yeast is added to this cooled sugar extract. The cultures of yeast are strains of *S. cerevisiæ*, and the greatest care is used by the brewer in keeping the yeast cultures free from foreign organisms. If serious contamination occurs, or if for any other reason the yeast starters lose their vitality, the brewer must either get a starter from another brewery or by culture methods obtain a fresh starter. The yeasts that produce beer are spoken of as bottom yeasts, as they grow at the bottom of the vat, while those producing ale are top yeasts. In a few days a violent fermentation occurs in the vats, and carbon dioxide is given off in large amounts, while alcohol is formed up to from 4 to 8 per cent. When the fermentation has reached its maximum, the brew is cooled to a low temperature and drawn into casks to undergo a slower fermentation from six to sixteen weeks or longer.

There are various abnormal fermentations called diseases of beer, which are due to the growth of foreign yeasts or bacteria. These growths are prevented by careful control of the temperature, by care of the starter, and by pasteurization of the final product.

The fermentation of wine or apple juice is essentially the same, except that wild yeasts naturally present on the fruits

are depended upon for fermentation. The percentage of alcohol obtained may be somewhat higher than in the case of beer. The fermentation of molasses or of any sugary or starchy solution may be depended upon to produce ethyl alcohol, and by distillation the percentage of alcohol contained in any beverage or fluid may be reënforced.

Apart from the use of fermented products as beverages, which may well be condemned, alcohol has various commercial uses. It is the readiest and cheapest of the fluid fuels, and with the exhaustion of natural sources of oil and gasoline we may anticipate that alcohol will ultimately find a place as a substitute.

Acetic acid, or vinegar, is a familiar condiment and preservative in the household. It ordinarily contains from 6 to 10 per cent of acetic acid (CH_3COOH). Acetic acid may be made from any solution which contains from 6 to 12 per cent of alcohol. If left for a while such solutions will ordinarily undergo spontaneous acetic acid fermentation. More than 14 per cent of alcohol acts as an antiseptic. The process is surer and more rapid, and the end product is of a much better quality, if a starter is used, such as a small quantity of old vinegar of good quality.

We may illustrate the production of acetic acid in the case of apple juice. If, after cider has become hard, it is freely exposed to the air, or if a bit of mother of vinegar is added to it, a scum or film will form on the surface, and in time the alcohol will be slowly converted into acetic acid. We may expect from 8 to 10 per cent of acetic acid, but beyond that point the acid itself becomes antiseptic for the bacteria. If the scum that we have mentioned is examined under the microscope, it is found to be composed of short rods, usually contained in long chains held together by a gelatinous matrix. Oftentimes the ends of the cells are swollen or even branched. These are the characteristic acetic acid bacteria belonging to the genus *Acetobacter*. Those most commonly met with are *Acet. aceti*, *Acet. pasteurianum*, *Acet. kützianus*, *Acet. acetigenus*. All these forms are strongly aërobic. They grow through

a wide range of temperatures, but ordinarily from 30° to 35° C. is the most favorable range.

Manufacture of vinegar. We have noted that vinegar may be made from any alcohol fermented product, as wine, beer, cider, or other sugar solutions. The preliminary fermentation of the sugar into alcohol has already been described somewhat in detail. The guiding principles in the manufacture of vinegar are relatively simple. They depend upon abundant free circulation of air, a

large exposed surface, and a support or float for the surface film. All these things, it is evident, are to provide the bacteria with abundant oxygen. If the manufacture is continuous, means for removing part of the acetic acid when it has reached its maximum concentration, and replacing it with an alcohol solution of the proper concentration with as little disturbance as possible, is essential.

For small-quantity production of vinegar in the home a jug or large bottle may be filled about one-third full, and a small quantity of old vinegar added. A few chips or bits of excelsior will serve as a support for the membrane to be formed. The jug or bottle is turned on its side and left uncorked, and is not to be disturbed for three or four weeks. Then the strong odor of vinegar will be apparent.

For production in larger quantities a cask may be used. Good-sized holes should be bored through each head, to allow circulation. A few chips are thrown in, and the alcohol solution is run in so as to fill it about half full. If the process is continuous, a hole in the lower part of one head is bored, and a

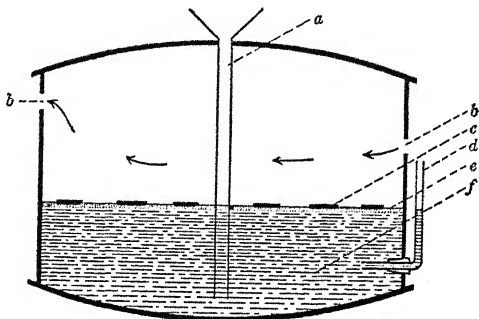


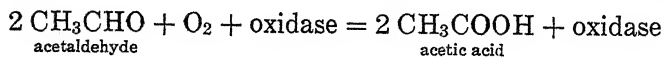
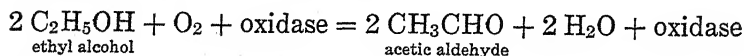
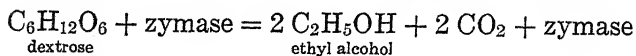
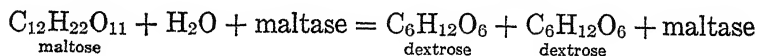
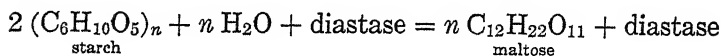
FIG. 38. Cask for manufacture of vinegar
a, funnel for introducing alcoholic solution; b, holes for circulation of air; c, floats to support growth; d, glass gauge; e, film of *Acetobacter*; f, fermenting fluid

cork with a bent glass tube inserted. This permits inspection as to the height at which the liquid stands in the barrel, and also allows withdrawal of the vinegar without disturbing the surface growth. Another hole through the top allows the insertion of a funnel well down to the bottom of the cask, through which new alcohol solution may be added.

A rapid method of commercial manufacture involves the slow trickling or the intermittent application of alcohol solution to a filter made of chips of beech wood, charcoal, or coke, which will support the slimy growth of the *Acetobacter*.

The diseases of vinegar. The vinegar eel (*Anguillula aceti*) is commonly seen in vinegar; it is offensive in appearance and may cause a disagreeable odor and flavor. These may be removed by filtering or by heating to 50° C. The vinegar fly (*Drosophylla cellaris*) may also be bothersome, but the same remedies are applied as for the vinegar eel. Other bacteria may cause trouble, and there are some that will even attack the acetic acid.

The chemical formulæ of the reactions involved in the various fermentations considered are illustrated below:



Lactic acid fermentation. Lactic acid ($\text{C}_3\text{H}_6\text{O}_3$) is one of the common end products of fermentation. Its most familiar source is in milk, it being the principal acid formed by the bacteria which sour milk. A very large group of bacteria produce lactic acid either with or without the production of gas. This property characterizes one of the large tribes of the

bacteria, the *Lactobacilli*, of which the buttermilk organisms *L. acidophilus* and *L. bulgaricus* are examples. Also an important group of the intestinal bacteria, which are commonly sought in the sanitary analysis of water, produce lactic acid and gas.

Many other carbohydrate substances undergo fermentation with the formation of various acids, as butyric acid and oxalic acid, each one of them depending upon a specific characteristic enzyme produced by a microörganism. In each case the principles involved in the study of the processes or the production of particular by-products of fermentation will depend upon a knowledge of the cultural requirements of organisms causing the particular fermentation.

CHAPTER XI

THE FERMENTATION OF NITROGEN COMPOUNDS

Bacteria of the soil. The nonnitrogenous organic compounds are relatively simple, and the fermentative changes caused by microbic enzymes acting upon them are relatively simple. The nitrogenous compounds, on the other hand, are exceedingly complex. The living tissues and cells, from the smallest to the largest plants and animals, contain protein and other nitrogenous substances. While the atmosphere that we breathe consists chiefly of nitrogen gas (79 per cent), yet in this form it is not available for either plant or animal life, excepting for two extraordinary groups of soil bacteria which we shall discuss presently. It is of supreme importance, therefore, that the nitrogen already found in available form for living things be kept within this chemical cycle and that it be kept in rapid circulation. The soil bacteria play a major rôle in this cycle, as well as in the carbon cycle already discussed.

The soil is veritably a "living earth." Wherever plants and animals thrive, there we find the soil alive with microorganisms: bacteria, fungi, and protozoa. The number of bacteria found in a gram of rich humus soil is almost incredible, running into the hundreds of millions. Usual quantitative methods, which show only the organisms that will grow under the particular culture conditions that we provide, reveal from five to fifty million. Bacteria are few or absent in the arid desert sands or in the perpetually frozen arctic soil. Their numbers decrease rapidly as we dig down vertically into the soil; and in the subsoils (clay, gravel, or hardpan) we may find sterile conditions. Plants do not thrive in such soils either; so, in general, we find microorganisms numerous in proportion to the other living things upon the earth.

The environmental factors influencing microbial life which have already been discussed are those which control the abundance of the microflora in soil.

Moisture is of prime importance. This depends upon the abundance of rainfall, evaporation, and drainage. The last two factors are directly influenced by the physical composition of the soil. The capillary water and hygroscopic water contained in the soil depend upon surface-tension phenomena. A soggy soil "sours" or becomes acid because of the activity of fermenting organisms and the restriction of other forms.

Aëration also influences microbial life in soil. This is best illustrated by reference to the changes that take place in sewage filters. Filters, when compact, or when dosage is continuous, yield a putrescible, unpurified effluent. Contact with a very porous bed, or intermittent dosage, results in oxidation and chemical stability of the decomposable substances in sewage.

Food material is another controlling factor of microbial life in soil. Food may be organic or, in some instances, may consist of inorganic salts. The kind and abundance of food material will largely regulate the abundance and kind of organisms.

Symbiosis in its broadest sense, including antibiosis and metabiosis, plays an important part. For example, a soil supplied with nitrates and a rich manure at the same time makes ideal conditions for the denitrifying bacteria. They use the organic material as a food supply, and the energy thus afforded makes possible the reduction of NO_3 , robbing the oxidizing bacteria of food. On the other hand, the ammonifying bacteria make possible the existence of the nitrous group; and the latter, in turn, furnish oxidizable material for the nitric bacteria. The whole cycle of changes brought about by soil bacteria is a closely associated phenomenon.

We should mention, too, the intimate association of certain microbes with higher plants. The legumes afford a desirable habitat for the nitrogen-fixing bacteria.

The soil is a product of the ages. The ceaseless action of rain, frost, tides, wind, and other physical forces, and the

action of living agents by the solvent action of their secretions or such physical forces as growing roots forced into rock crevices, have worn down the primitive rocks. The dead bodies of plants and animals have accumulated and have been decomposed by microorganisms until over a large part of the earth's surface we have a thin covering of soil rich in organic matter which we call humus. Many salt constituents are essential for plant growth, but ordinarily the chemical composition of humus is satisfactory for continuous cropping except for phosphorus compounds and nitrates.

Bacteria hasten the return of phosphorus to the soil in available form by decomposition, and also convert the insoluble phosphates into soluble form by the action of organic acids and carbonic acid which they form. The presence of phosphates, in turn, favors the rapid growth of other bacteria favorable to the soil. The bacteria have no power of actually adding phosphates to the soil, and the impoverishment of these compounds leads to the necessity of adding them in the form of soluble salts, of which there are only limited natural sources.

With nitrogen the situation is different; for not only does barnyard manure contain a large amount of this element, found in a form which the bacteria can work over and render available for plant food, but, further, the bacteria, whether free in the soil or in the roots of certain crops like clover or soy bean, may actually *fix* or oxidize atmospheric nitrogen. The chemist can perform this difficult feat by the use of a powerful electric current, and in nature oxides of nitrogen are formed in the atmosphere by the discharge of lightning. Bacteria carry on the work in a quiet, efficient way, day and night, when soil conditions or cropping favor their growth.

Classes of soil bacteria. In their entirety these groups include all the types of decomposition and synthesis. The chief groups are the *carbohydrate-fermenting bacteria*, which act upon carbohydrate substances (cellulose, starch, and sugars); the ammonifiers, which decompose nitrogenous compounds, yielding finally ammonia; the nitrifiers, which oxidize ammonia to nitrite and then nitrite to the nitrate stage; the denitrifiers,

which reverse the cycle and reduce NO_3 to NO_2 , and the latter to NH_3 and free N_2 gas; and, finally, the nitrogen-fixers, which seize upon the atmospheric nitrogen gas and build it into their own complex proteid substance.

Carbohydrate-fermenting bacteria. The chief constituent and most stable substance of the plant body is cellulose. The filamentous fungi, as well as the bacteria, are important agents in rendering this substance soluble and producing from it carbohydrate food of simple form for other organisms. The enormous amount of cellulose in the soil, and the enormous source of energy represented by it, make the importance of this group of microorganisms obvious. Most of the carbon synthesized by the green plants appears as cellulose. A great many of the bacteria acting upon cellulose are anaërobic. Little is known about them as a group, and their importance is often overlooked because of their presence and abundance in nearly all soils. Their action is taken as a matter of course.

A great many species of bacteria ferment the less complex starches and sugars, yielding organic acids and, as end products, methane, hydrogen, and carbon dioxide.

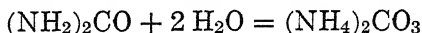
Peptonization and ammonification. A great number of widely dispersed species of soil bacteria are able to attack the complex plant and animal proteins. Proteolysis, or the initial splitting of the large molecule by the addition of water due to the proteolytic enzymes of the bacteria, is the first step. Successive cleavages into proteoses, peptones, polypeptids, and, finally, amino acids occur. These changes are spoken of as peptonization. The amino acids are attacked by bacteria and yield chiefly ammonia. This is known as ammonification. Reference to the diagram (Fig. 39) will make this clear. Ammonia serves in a limited way as a food for plants, especially the cereals, rice preferring ammonia to nitrate; so that the organisms involved really prepare a plant food.

The aërobic spore-formers — *B. subtilis*, *B. mycoides*, *B. megatherium*, and so on — are universally found in soils, and they are ammonifiers. However, they occur in soils chiefly in the resting, or spore, state and have been shown to grow

actively in soils only under special conditions which do not ordinarily obtain. On the other hand certain non-spore-formers, *Pseudomonas fluorescens* and *Pseudomonas caudatus*, are also widely distributed and multiply rapidly in manured soils. Probably the species involved in ammonification in natural soil are very numerous. They are universally present, and no special soil inoculations are necessary. Those conditions which favor plant crops, as manuring and cultivating to keep the soil loose and well ventilated, likewise favor the ammonifiers. Though less spectacular than the nitrifiers (to be considered presently), their activities are no less essential.

Soil fertility is determined largely by the readiness with which proteolysis and ammonification take place, and it is possible to test soils in the laboratory in this respect by analyzing manured and incubated soils for their ammonia content.

During life, animals give off waste nitrogenous products in the excreta. The most prominent compound is urea, $(\text{NH}_2)_2\text{CO}$. A very important group of bacteria have the ability to ammonify urea by a simple hydrolysis:



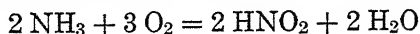
There seems to be no organic nitrogenous compound that may not be ultimately broken down into ammonia by some soil microörganism.

Barnyard manure is the commonest form in which nitrogen is applied to the soil. Ammonification occurs in the manure pile, and much is lost as a free gas, because the acids and salts which fix the ammonia are largely absent. There is obviously a great economic value in applying manure promptly and in mixing it with garden soil. Tight packing and covered manure pits to prevent the escape of CO_2 , which reduces the volatilization of the ammonia, are an advantage. Enormous losses occur by the ordinary old-fashioned methods of storing and handling manure; and with the continuing impoverishment of the soil and the increasing demand for nitrogenous foods there is every reason for a more thorough knowledge and application of scientific methods.

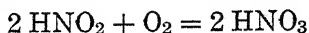
Nitrification. We have noted that ammonia has a limited use as a plant food. Nitrate salts are the principal source of nitrogen for the green plants. Unless nature provided for the oxidation of ammonia into the nitrate state the life of higher organisms would soon cease. As has been anticipated, nature has not been remiss in this respect, and we find an extraordinary group of prototrophic bacteria that are able to oxidize ammonia in two distinct steps — through the nitrite into the nitrate state.

It was recognized before the days of biology that nitrification in the soil must occur, as analyses showed that when fields were fallowed, or remained unused, the nitrate content of the soil increased. It was thought that this was a purely chemical reaction; but some fifty years ago, during the time when the practical applications of microbiology were first being made, it was discovered that sterile soils did not show any increase in nitrogen. Winogradsky was the first one to show that certain species of bacteria were definitely involved in this oxidation process.

The early results were confusing because it was not realized that two distinct groups of organisms were involved: the one able to transform ammonia into nitrite, which incidentally is never a plant food but in appreciable concentrations acts as a plant poison; the other oxidizing the nitrites into nitrates. The first process is known as nitrosation, and is carried on by a group of bacteria belonging to the genus *Nitrosomonas*, according to the following formula:



The second stage of oxidation is called nitration, and the bacteria involved belong to the genus *Nitrobacter*. The formula for this reaction is



The nitrifying bacteria, as a group, may be described as usually short rods, sometimes spherical forms, not producing spores, and strongly aërobic. They are present in nearly all

soils that are not too acid. The conditions of the soil that encourage their action depend upon the physical nature of the soil, the most important elements being good aëration and the presence of sufficient moisture.

While in the laboratory organic matter inhibits the growth of this group of bacteria, this is not so in well-cultivated soil. It is evident from what has been said that those conditions which ordinarily encourage crops are likewise favorable for the growth of this group of organisms. The fallowing of soil, which has been popular in the past, is no longer considered good soil practice, unless under exceptional conditions where the water content is insufficient to allow of continuous cropping.

While it is probable that other bacteria, and perhaps molds, may be capable of nitrification, their action is incidental, and it is unquestionably true that the *Nitrosomonas* and *Nitrobacter* groups are of transcending importance as nonsymbiotic nitrifiers.

Denitrification. Up to this point the action of the various bacteria of the soil has been beneficial to organic nature. There is, however, a group of organisms which cause distinctly unfavorable chemical changes. In fact, they directly reverse the processes that have just been described, reducing nitrates to ammonia and even liberating nitrogen as a free atmospheric gas, thus removing it completely from the organic cycle.

Denitrification takes place in separate steps and may go to completion, liberating nitrogen gas; or it may be what is called partial denitrification. It is evident that a partial denitrification is not so serious a matter, as it may be counteracted by the action of the nitrifiers. In fact, under certain conditions the nitrifiers themselves may actually reduce nitrates. The loss of nitrogen gas is a more serious matter.

Since these reactions are all reduction processes, no energy is derived from them. The soil conditions that encourage denitrification are the presence of abundant, readily available organic matter, including carbon compounds, and anaërobiosis.

The denitrifying bacteria do not belong to any special group, but there are a large number of organisms in soil,

most value in sewage, and sanitary engineering has pointed a way to at least a partial solution of the problem through scientific treatment of sewage.

Nitrogen fixation. The losses of nitrogen gas by denitrification are fortunately counterbalanced by another activity of the bacteria. Among the most remarkable of all living things is this group of organisms, which actually take atmospheric nitrogen and oxidize it, or *fix* it, in such form that it becomes available for the food of green plants. It is possible for the chemist to fix nitrogen in the laboratory, and potassium nitrate can be produced commercially by the use of a powerful electric current. But the cost of production is enormous compared to the cost of letting bacteria increase the fertility of the soil while it is at the same time at work yielding crops.

The nitrogen-fixing bacteria belong to two very distinct genera: one of them living free in the soil and capable of fixing nitrogen by the use of energy derived from carbohydrate foods; the other form living as a symbiont, or parasite, within the roots of certain of the *Leguminosæ*. Berthelot first recognized the fact that nitrates increased in certain barren and sterilized soils. In 1893 Winogradsky proved that microorganisms were associated with the process, when he isolated and cultivated an anaërobic organism, which he called *Clostridium pasteurianum*. It is now well established that a group of aërobic bacteria, called the genus *Azotobacter*, are present in most soils and that under favorable conditions they will produce nitrates, or, rather, will fix atmospheric nitrogen. The presence of certain salts, notably calcium and magnesium carbonates, are of importance, and the amount of the nitrification taking place in the soil may be used as an indirect measure of its salt requirements.

Cultivation, to keep the soil porous and to retain its moisture content, likewise favors the activity of these organisms. It might be supposed that soil inoculation would be possible with the organisms belonging to this group. Although this has been tried it is generally true that, given suitable cultural conditions in the soil, these organisms are usually present and

will thrive. Attention, therefore, should be devoted more to the condition of the soil which fosters this group of bacteria than to methods of inoculation.

Symbiotic fixation. The rotation of crops is a very old practice, and it has generally been recognized that one of the legumes should be included in the rotation of crops. Ward (1887) demonstrated that in little tumors or nodules, that had previously been described, there were living bacteria that did not develop in the roots if the plants were grown in sterilized soils. And, further, he proved that plants could be inoculated with these bacteria by bits of tubercle transplanted from the roots of other plants. The bacteria found in the nodules are minute rods which characteristically produce cells with swollen ends, frequently assuming the shape of *y*'s or *t*'s. These are spoken of as bacteroids, and appear in the root nodules after they are well developed. The organisms belong to the genus *Rhizobium*. Although they may live free in the soil and even fix nitrogen under these conditions, they are more commonly found associated with the roots of plants. They enter through the tiny root hairs, being attracted, apparently, by certain secretions from the roots. They break down the wall, and a tiny tube develops into which the bacteria stream. A stimulant is produced by the bacteria which results in cell proliferation, and we get the tumors or nodules. Apparently the bacteria derive their carbohydrate food from the plants; and the nitrogen gas, brought to them by the circulating fluids in the cells, is directly oxidized. The plant, in its turn, seems to produce antibodies, which ultimately arrest the rapid growth of the bacteria, and to derive benefit from the presence of these invading organisms. Although this relation is commonly spoken of as symbiotic, it would seem more appropriate to call it a reciprocal parasitism. The bacteria infect the living cells of the plant and cause an abnormal cell proliferation which appears as nodules or tumors. The upper hand is held for a time by the bacteria, whereupon they succumb to the reaction on the part of the host, which may be considered as analogous to an immunity reaction.

The question of species among these bacteria is an interesting one, and it is usually granted that there is but one species which is susceptible to marked changes in its invasive and disease-producing ability on different plants. A strain transplanted from crimson clover to the soy bean will gradually increase its virulence for the latter plant.

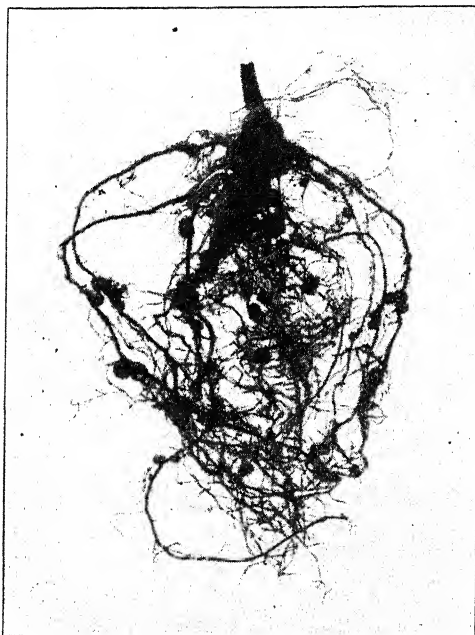


FIG. 40. Nodules on the roots of soy bean¹

Gains in the nitrogen content in soil that is cropped with legumes properly inoculated may run from one hundred fifty to over two hundred pounds to the acre. The crop may be plowed under, which is known as green manuring; but preferably the green should be harvested, plowing under only the root and stubble, and returning the nitrogen in the green crop to the soil, after feed-

ing, as barnyard manure. The animal manures are more beneficial to the soil than are green manures, and also, by this method, the crop has a dual use as a feed for stock and as a manure. It is entirely feasible to practice artificial inoculation with *Rhizobium* cultures. This is not always desirable if the soil has recently been cropped with the legume to be planted, or if it is definitely known that nodules are readily formed. With soil not recently rotated with legumes, or in poor soil, it is desirable. Soil transfer is one possible method — applying two to

¹ From *Farmers' Bulletin No. 1496*, United States Department of Agriculture.

five hundred pounds of soil known to contain the culture to the acre. This involves unnecessary labor and expense, and one may now obtain cultures commercially or from agricultural experiment stations in such form that they can be applied directly to the seeds, knowing in advance that the virulence of the culture is satisfactory. The seeds are immersed in a suspension of the culture, ordinarily, and are dried without being exposed to the sun, and are then promptly planted. This brings some of the organisms into direct contact with the soil surrounding the roots of the developing seedling.

While our knowledge of agricultural bacteriology is still far from complete, nevertheless we have become sufficiently familiar with the principles involved so that the scientific agriculturist can no longer afford to ignore its importance. Nitrification and nitrogen fixation seem to offer ample resources of nitrogen for crops in the future if we provide soil conditions for their cultivation and if we crop scientifically.

Sewage treatment. We have referred to the enormous waste due to the usual methods of disposing of municipal sewage. The problem of sewage disposal involves not only economic considerations but is of prime importance as a sanitary consideration; and the latter has been the principal concern of engineers up to the present time. Where artificial purification has been practiced, however, it has been learned that bacteria are the chief if not the sole reliance for changing the putrescible, unstable matter in sewage into stable form, and the chemical changes involved are essentially those which have been described in connection with the action of the proteolytic and nitrifying bacteria. The two general biological principles involved in sewage treatment are those which encourage the action of the powerful proteolytic anaërobes and those which encourage the growth of the proteolytic aërobes and the nitrifiers. The solid residue in sewage, called sludge, may be removed from suspension by the action of gravity when the rate of flow of sewage is sufficiently retarded, and it may be stored in large tanks or vats until it undergoes hydrolysis. Until recently it has been supposed that strict anaërobic conditions

best fostered such hydrolysis, and tanks were designed in such a way as to prevent the mixing of fresh sewage with the old sludge. It is true that a powerful dissolving action occurred under these conditions, and that a more or less peaty, non-putrescible residue was finally produced. More recently it has been learned that the same results may be obtained by bubbling air through the stored sludge, and that in this way the solid material finally recovered will contain a relatively high per cent of nitrates, which may be successfully returned to the soil. This method has been found so practical that at the present time the city of Chicago has constructed a unit to provide for the treatment of the sludge of eight hundred thousand people, by this activated method, at a cost of over \$27,000,000.

Considerably more than one half of the decomposable materials in sewage are held in solution as dissolved material, so that the removal of the solids is only a partial solution of the problem. This liquid waste containing decomposable nitrogenous organic matter may be applied to specially prepared soil areas, which are constructed and operated so as to give a maximum of ventilation. Aërobic nitrifying bacteria grow on the grains of sand or on the rocks composing the filter beds, and they rapidly turn the nitrogenous material into the mineral form of nitrates; so that the effluent of such filter beds contains little or no ammonia and large quantities of nitrate, and is therefore nonputrescible. These remarkable changes in the organic constituents of sewage occur during a contact of a few hours by the trickling of the sewage over rock beds no more than eight feet in thickness. This shows what amazing potentialities the bacteria have for performing their work if suitable culture conditions are provided.

CHAPTER XII

THE BACTERIOLOGY OF WATER

Importance of water to life. Water is of prime importance to life. Life may continue dormant in seeds and resting spores, but in the active state it requires free water. Human life is dependent upon an ample intake of water; and while we may go for weeks without food, man survives only for a few days without water. Modern living, and urban life with its manifold sanitary and industrial demands for water, require large amounts, every drop of which must be safe for drinking purposes. American cities require from ninety to a hundred gallons of water per capita per day as a minimum, while European cities have adapted themselves to a somewhat lower consumption. The accelerated growth of urban populations and the increase of people in the adjacent land on the natural watershed have demanded not only ever greater amounts of water but involve ever greater difficulties in maintaining its purity. The maximum density of population will probably be determined by water supply rather than food supply; for with financial ability a city can command the world's resources of food, but is dependent upon a restricted territory for its water.

The knowledge that water plays a large part in the spread of certain epidemic diseases, notably the acute intestinal infections such as typhoid and cholera, and the necessity for vast quantities of water which are increasingly exposed to the risk of contamination from sewage, has greatly accelerated the development of sanitary engineering and methods of testing water to obtain ample and reliable supplies of this indispensable commodity. The small household supply from the neighboring spring or well is of no less importance from the standpoint of health, and its protection and testing are essential.

Microorganisms in water. Microorganisms of a wide variety abound in waters. Their number and variety are determined chiefly by the amount of organic matter and salts in solution. Protozoa, algæ, and many fungi and bacteria find their natural habitat in water. The first three groups may affect the appearance or the odor and taste of water. Their presence may become so offensive in uncovered reservoirs that means must be taken for their elimination. This may be accomplished by the application of very small amounts of copper sulphate or by filtering and aërating the water. At least one protozoan, the amœba (*Endamœba histolytica*) causing one form of dysentery, may be spread by drinking water.

The bacteria in water are the most numerous and by far the most important microorganisms. Not only are there a number of groups that characteristically live in water, but many enter from outside sources: from the air, the soil, and the surfaces of plants washed by rain water; from the surfaces and alimentary wastes of animals; and, most important, from sewage of human origin. While the number and variety of bacteria found in any water may represent members from any or all of these sources, sanitary tests ordinarily treat as incidental all except bacteria of alimentary origin, because all the known infectious diseases spread by water are from this source only.

Kinds of bacteria in water. Natural waters are commonly classified according to their origin, as ground and surface waters. Shallow-dug wells, deep-bored or driven wells, and springs are ground waters; while streams, rivers, ponds, and lakes are representative of surface waters. Bacteria are found in all these, but the numbers and kinds ordinarily vary with the source.

We may group the bacteria that are found as (1) typical water bacteria; (2) those from all foreign sources except human drainage, including the air and soil; (3) those from drainage of human origin.

The water bacteria may be characterized in a very general way as non-spore-forming rods, not fermenting lactose, but

often liquefying gelatin, forming small colonies on laboratory media which may or may not be fluorescent or chromogenic. A few representatives of the Spirillaceæ are found in water, but almost never cocci. The water bacteria grow best, as would be expected, at room temperature or lower. The analyst never detects all the bacteria present in any sort of sample, so that the number of bacteria revealed by a water analysis is always less than the actual number present. Organisms from the soil or from animal excreta grow more readily under laboratory conditions, and the water bacteria are often concealed and overgrown. In ground water, in mountain brooks, and in lakes not recently roiled by rains we may expect to find true water bacteria. Their numbers are usually relatively small. We may find from one or two to a few hundred bacteria per cubic centimeter in such waters.

Bacteria from the natural wash, and currents due to melting snows and rain, are chiefly characteristic of the soil. The most prominent are apt to be the large, spore-forming, vigorously growing rods, strongly proteolytic but seldom fermenting lactose or other sugars. Many other varieties may be present and concealed by the more vigorous growth of these forms which flourish under laboratory conditions.

Bacteria representative of drainage from human sources, notably sewage, contain principally organisms of the intestinal tract. The large lower portion of the alimentary tract, the colon, serves the function principally of storing the unused food materials and the secretions poured into the upper canal. In the healthy stomach and small intestine, conditions are unfavorable for the growth or survival of microorganisms; but in the colon we find almost ideal conditions for a great variety, and the feces contain many millions to the gram. The principal microbe, and the one of transcending importance to the sanitary bacteriologist, is the colon group, of which *Escherichia coli* (*Bact. coli*) is the chief and typical representative. These organisms are normal inhabitants of the intestinal tract of warm-blooded animals generally; and because of the unlikelihood of their being derived in large numbers from any

source except sewage, their presence in water in appreciable numbers is an almost sure indication of dangerous contamination. The general characteristics of the tribe *Bacteriaceæ*, to which this group of organisms belongs, are as follows :

Gram-negative, evenly staining rods occurring commonly in the intestines of animals. Often motile with peritrichic flagella. Easily cultivable, forming grape leaf or convex whitish surface colonies. Liquefy gelatin rarely. . . . Acid formed by all ; gas (CO_2 and H_2) only by one series. Typically intestinal parasites of the higher animals, although several species may occur on plants and one (*Aero. aërogenes*) is widely distributed in nature. Many species pathogenic.

Fig. 41 shows the general relations of the more important species of the colon-typhoid group, as to their ability to attack carbohydrates.

For preliminary identification of characteristic *Esch. coli* in water analysis, Gram-negative, non-spore-forming bacilli which ferment lactose with gas formation in appreciable amounts, and which grow aërobically on solid media, are sufficient evidence.

Besides members of this group eliminated from normal persons, there are pathogenic forms, including *Eberthella typhi*, *Salmonella paratyphi*, *Shigella dysenteriae*, and others. It is evident that their distribution in nature will be coextensive with the more common colon organism ; for they grow exclusively in the human intestine, and the finding of *coli* will at once arouse suspicion of the presence of pathogenic forms. It would be gratifying actually to isolate and identify the typhoid organism from water in order to have proof positive that a particular outbreak was spread by water, for example. Such isolations have been made. The technique is exceedingly difficult and unreliable ; and since raw sewage in water is potential evidence of typhoid, the indicative tests for *coli* are sufficient.

There are many bacteria other than representatives of the typhoid-colon group present in sewage. Cocci abound in the alimentary canal. They are usually spoken of as *sewage streptococci*, although long-chain forms are not especially

prominent. They grow best in the presence of sugars and on solid media containing sugar, especially lactose; and they form subsurface colonies with acid but no gas. Their presence in water is usually considered to be indicative of recent pollution, since they do not survive as long as *Bact. coli*. By

	<i>A. aërogenes</i>	<i>A. cloacæ</i>	<i>A. neapolitanus</i>	<i>E. communior</i>	<i>E. coli</i>	<i>E. acidilactici</i>	<i>S. schottmülleri</i>	<i>S. enteritidis</i>	<i>S. supester</i>	<i>S. paratyphi</i>	<i>Eb. typhi</i>	<i>Sh. dysenteriae</i>	<i>Sh. shigæ</i>	<i>Alc. facidis</i>
Hexoses	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+

FIG. 41. This diagram shows the fermentative relationships of the more important species of the colon-typhoid group¹

Note how few gaps there are in reading up and down, or from left to right

differential fermentation it is possible to determine whether they are from the human intestine or from domestic animals. In America the test for cocci is usually ignored.

Another group, the lactose-fermenting, anaërobic spore-formers, as *Clostridium welchii* or *Clostridium sporogenes*, are numerous in sewage; because of spore production they will

¹ Winslow, Kligler, and Rothberg, in *Journal of Bacteriology*, Vol. IV (1919), No. 5, p. 493.

survive the longest of all the sewage bacteria, and may give an indication of remote pollution.

The colon test by itself is sufficient and gives the information desired about the sanitary nature of water, so that the recommendations of the committee of the American Public Health Association include only two principal tests: (1) those for the quantitative determination of bacteria by the plate count on gelatin incubated for forty-eight hours at 20° C. and on agar incubated for twenty-four hours at 37° C., and (2) the "presumptive" and "confirmed" tests for *Esch. coli*. The presumptive test is one giving evidence of the sought-for organism directly from the water sample. Since there are some bacteria which occur, though rarely, in water, and which give the same preliminary reaction, it may be necessary to run further tests on the isolated organism, to give ample confirmation of this presumptive evidence. These are the confirmatory tests.

All these organisms seem to be confined to the alimentary tract of warm-blooded animals, chiefly man, as a natural habitat, with the exception of *Aërobacter aërogenes*, which has been isolated from soil and grains and other uncontaminated natural sources.

The following table, compiled from the summary made by Levine,¹ shows the findings of a number of investigations:

TABLE IX. THE RELATIVE FREQUENCY OF OCCURRENCE OF AËROBACTER AËROGENES AND ESCH. COLI FROM DIFFERENT SOURCES

SOURCE	NUMBER OF WORKERS	NUMBER OF STRAINS ISOLATED	PERCENTAGE OF A. AËROGENES	PERCENTAGE OF E. COLI
Human feces	13	2534	5.9	94.1
Animal feces	11	1832	7.4	92.6
Soil and grains	8	1141	86.5	13.5

It is seen from this that *Esch. coli* is preponderantly an intestinal form, and *A. aërogenes* preponderantly a soil saprophyte, although they may occasionally be found overlapping in the same environment.

¹ M. Levine, in *American Journal of Public Health*, Vol. XI (1921), p. 21.

Although in the presumptive test *Esch. coli* and *A. aërogenes* are indistinguishable, they may be readily differentiated after isolation. Three tests known as the Methyl Red, the Voges-Proskauer, and the Sodium Citrate Test seem to be sufficient, although Standard Methods¹ adds several other tests, supposed to distinguish between *A. aërogenes* of fecal and nonfecal origin. The methyl red test depends upon the hydrogen ion concentration produced by the respective organisms in dextrose dipotassium hydrogen phosphate broth, as revealed by the addition of five drops of the indicator to the incubated culture. This gives a distinct red with *Esch. coli* at the higher hydrogen ion concentration (methyl red +) and a distinct yellow with *A. aërogenes* (methyl red -). The Voges-Proskauer test is performed with a portion of the same medium, by adding to 5 cc. of the incubated culture 5 cc. of a 10 per cent solution of potassium hydroxide. After shaking, or allowing to stand overnight, a positive test is indicated by the appearance of an eosin-pink color. This is developed in the *A. aërogenes* culture (V.-P.+) but not in the *Esch. coli* culture (V.-P.-). The test in sodium citrate medium is merely for the presence or absence of growth, coli being unable to utilize carbon from this source. To summarize in this table:

	METHYL RED	VOGES-PROSKAUER	SODIUM CITRATE
<i>Esch. coli</i>	+	-	-
<i>A. aërogenes</i>	-	+	+

All waters of unknown quality, whether for a community or for a household, should be tested before they are accepted for use. Occasional tests are sufficient thereafter for small local supplies; but municipal waters should be tested daily, to detect any fluctuations and to give immediate warning of any danger. Waters that are purified by any method should be tested in a routine way, to check on the performance of the filter or disinfecting devices, as well as to prove that the water is continuously safe.

¹Standard Methods of Water Analysis. American Public Health Association. See Appendix B.

Of the eighty-three cities with a population of a hundred thousand and over which were examined, practically all provided for regular laboratory control of the water supply.

Interpretation of results. After the laboratory tests have been made, how shall they be interpreted? What constitutes a safe water in terms of bacteriology? Briefly, we may say, one that is entirely free from lactose-fermenting bacteria of sewage origin. In practice the answer is not so simple. A majority of raw water supplies today would show the presence of *Esch. coli* if we could examine large enough amounts. They may not be dangerous if such organisms are present one in ten liters, for example. Standards must be set as to the permissible numbers that may be present in a safe potable water. We should know also the limitations of filters and disinfection, and what waters may be rendered safe by these treatments.

It is difficult to set up empirical, arbitrary standards in this respect. The trained person would take many factors into consideration in rendering an opinion: the season, the sanitary inspection, the numbers and kinds of bacteria, etc. From the quantitative standpoint we ordinarily expect relatively low numbers of bacteria in ground waters. Hundreds or thousands arouse suspicion of surface drainage and the presence of organic matter, regardless of other factors. The same is true of mountain brooks and of clear impounded waters or natural lakes. Properly collected samples, promptly analyzed, give not over one or two hundred bacteria per cubic centimeter at the outside. The plates incubated at body temperature, especially, should give low counts. River waters, on the other hand, are subject to much wider fluctuations, and, regardless of sewage pollution, may safely contain several hundred to a few thousand bacteria. This seems to be explained by the fact that the currents keep whatever bacteria may enter the water buoyed up, while in quiet waters they rapidly sink to the bottom. Other factors play a part, too.

The more significant tests for sewage bacteria have a definite basis. Infectious agents are potentially present in proportion to these organisms.

Arbitrarily we may condemn any water having *Esch. coli* present in the majority of 1-cubic-centimeter samples, and accept a water as safe if this organism is uniformly absent in 100-cubic-centimeter amounts. The border line of safety comes somewhere in between. Ten-cubic-centimeter portions are taken as the standard amounts to be examined. Generally speaking, *Esch. coli* should be absent from the majority of the standard portions tested.

The Report of the Advisory Committee on Official Water Standards appointed by the Surgeon-General of the United States¹ gives the following bacteriological standards:

1. Of all the standard (10 cc.) portions examined in accordance with the procedure specified below, not more than 10 per cent shall show the presence of organisms of the *Esch. coli* group.

2. Occasionally three or more of the five equal (10 cc.) portions constituting a single standard sample may show the presence of *Esch. coli*. This shall not be allowed if it occurs in more than

- a. Five per cent of the standard samples when twenty (20) or more samples have been examined.

- b. One standard sample when less than twenty (20) samples have been examined.

This standard apparently attempts to set the mean permissible density of *Esch. coli* in drinking water as not greater than 1 per 100 cubic centimeters, allowing for the action of the laws of chance when a small number of samples are tested.

Raw waters are frequently unsafe to use without preliminary purification. This is increasingly true as populations become more dense on the watersheds.

The three principal methods of purification are (1) storage, or impounding, to allow subsidence and other natural forces to act; (2) filtration through specially prepared areas of well-drained sand, either with the addition of a flocculating chemical (rapid filtration) or without the addition of any chemical (slow filtration); (3) disinfection, usually by the use of chlorine in some form. Either method of filtration, when performing properly, should remove from 95 per cent to 99 +

¹ Public Health Reports, Vol. XL (1925), No. 15, p. 696.

per cent of all bacteria. The percentage of removal will vary with the bacterial content of the raw water, being higher when the numbers are high and lower when the numbers are small. Reference to the table shows the results of treating Ohio River water at the purification plants of a number of cities using this supply. In these plants the water is subjected to settlement, rapid sand filtration, and chlorination.

TABLE X.¹ AVERAGE OF MONTHLY MEAN LABORATORY RESULTS REPORTED FROM INDIVIDUAL OHIO RIVER PLANTS DURING THE PERIOD OF OBSERVATION

CITY	BACTERIAL COUNT PER C.C. (AGAR, 48 HRS. 20° C)					ESCH. COLI INDEX PER 100 C.C.					PERIOD
	Untreated Water	Primary Settled	Applied to Filters (coagulant)	Filtered	Chlorinated	Untreated Water	Primary Settled	Applied to Filters (coagulated)	Filtered	Chlorinated	
Steubenville .	1,650	1,580	379	248	79.0	333	160	55	.6	.2	July, 1923— June, 1924
Ironton . . .	19,600	1,790	476	28	4.0	14,900	1,460	162	1.6	.1	July, 1923— June, 1924
Portsmouth .	9,910	1,270	778	73	.5	3,490	344	79	1.7	.1	July, 1923— June, 1924
Cincinnati .	18,300	2,580	464	65	31.0	2,980	575	85	3.4	.4	July, 1923— June, 1924
Louisville . .	12,500	13,200	2,360	2,270	12.0	2,220	1,140	135	17.0	.1	Sept. 1923— Aug. 1924

Other tests for water. Water may be tested for its physical and chemical properties as well as for its biological content. At the outset we demand water that is clear, colorless, cold, and without disagreeable taste or odor. Aesthetic standards require this, although there may or may not be a relation between these tests made by the senses and the safety of the water.

Chemical tests for organic matter, notably to determine the condition and amounts of nitrogenous matter, and for certain inorganic salts such as chlorine, are of more significance, but are far less indicative of specific sewage contamination than is the finding of bacteria always associated with sewage.

Tests or observations of the source of a water, if made by a trained person, are exceedingly important. This sanitary in-

¹ Public Health Bulletin No. 172, Treasury Department, United States Public Health Service. Condensed Table.

spection often tells the story without refined laboratory tests, and the tests usually confirm the field observations. With household sources, as wells and springs, this is particularly true. Wells, improperly constructed, with loose stone lining and board covers, or dangerously located near privy vaults, or in the barnyard, where they will receive surface drainage and seepage, tell their own story. Often, however, the source of contamination is more subtle, and the first warning comes from the laboratory. With large supplies coming from rivers or large lakes, or impounding reservoirs with vast watersheds, the sanitary inspection requires very comprehensive study. The increase in picnickers and auto campers has menaced many a spring or roadside brook, due to lack of ordinary sanitary sense on the part of the users. The thermos, with a supply from home, or tea or coffee made from the boiled water, is a safer drink.

Ice. Ice, being frozen water, often harvested from natural ponds and rivers, must have the same potential bacterial content as the water from which it is formed. Analysis of melted ice usually shows a relatively low bacterial content. In forming, especially if the surface is quiet, many bacteria and other foreign suspended matter are frozen out; while of those caught in the crystallizing water, many are killed instantly. In snow ice, or ice containing bubbles and foreign matter, bacteria escape this crushing and thereafter die off slowly. That some survive in ice for a matter of many months has been shown, and experimentally the typhoid bacillus has lived for three weeks. The usual storage of several months before natural ice is used is an obvious safeguard. These considerations explain why ice taken from sources unfit for water supplies is ordinarily rendered safe.

With artificial ice there is no mode of escape for the bacteria in the congealing cake, and they are usually left concentrated in the last fluid water to crystallize. This might increase the risk somewhat, except that the water used in the ice plant is ordinarily from the same source as the community

supply, and that the small amounts used can easily be subjected to artificial purification.

Shellfish. The bacteriology of shellfish is closely related to the microbic content of the water from which they are harvested or in which they are subsequently bathed. The bacteriological tests of shellfish are modifications of those used for water, being essentially a search for *Esch. coli* in samples of shell water or stomach contents. This material undiluted and diluted is inoculated into lactose broth fermentation tubes, and the formation of gas is considered presumptive of sewage bacteria, to be followed by confirmatory tests if necessary. The presence of *Esch. coli* in each portion tested is given a value as follows:

Present in 1 cc., but not in .1 cc. = 1

Present in 1 cc., but not in .01 cc. = 10

Present in .01 cc., but not in .001 cc. = 100

Five samples of each dilution are tested, and the values added to give a final score. The following table gives a hypothetical example of the way the score is obtained:

TABLE XI

SAMPLE	1 CUBIC CENTIMETER	0.1 CUBIC CENTIMETER	0.01 CUBIC CENTIMETER	NUMERICAL VALUE
1	+	—	—	1
2	+	+	—	10
3	+	+	—	10
4	+	—	—	1
5	+	+	—	10
			<i>Final Score</i> 32	

The familiar slogan which appears in the fish store in September, "Oysters R in Season," — suggesting that the months with *r* in their spelling, the cold months, are safe for shellfish, — has a bacteriological justification; for there is a definite increase in bacteria in May and during the summer months,

and a decrease in the fall. This is explained by the feeding activities of oysters. They tend to hibernate as the water cools, and actively feed upon any sort of suspended organic matter, including sewage and bacteria, as soon as the water warms up.

Preventing the contamination of sea water which bathes shellfish areas, prohibiting the practice of "floating" oysters in polluted fresh or brackish waters, and carefully regulating and inspecting the handling and shipping of shellfish will minimize the danger of infection. Shellfish will "purify" themselves if they are removed from polluted to pure water and kept there for a few weeks, and they may also be disinfected by being exposed to circulating sea water containing chlorine. The check on the practice lies in the laboratory tests.

CHAPTER XIII

THE BACTERIOLOGY OF MILK

Importance and uses of milk. Next to water, milk is unquestionably the most universal and essential food that man uses. Starting with the first day of life, up until the end, the daily diet of man usually includes milk. The only natural food for the baby during the first six or nine months of life is mother's milk. Cow's milk is sooner or later substituted for human milk and should be the chief article of diet through the fourth or fifth year of life. The use of at least a quart of milk a day all during childhood, and its liberal use throughout life, is urged by most dietitians. The convalescent is nursed back to health and strength on a diet liberal in milk. As accessories in coffee or tea, on cereals, and in all sorts of cooking, milk and cream are used. Add to this the use of butter, ice cream, cheeses, and other products exclusively or chiefly made of milk, and one begins to realize the importance of this food.

In a young, pioneer country or in the agricultural and rural parts of the land, the milk supply is near at hand, and the pail of fresh, warm, frothing milk is a familiar sight. Milk a few hours old, poured from the pail into the bottle, is delivered, and perhaps used the same day it is produced. It is handled by one or two persons and passes through only two or three receptacles before it is used. While this does not necessarily imply that the milk is either clean or safe, it does mean that the risks of bacterial contamination and growth are greatly minimized as compared with the vast and complicated problem of the milk supply of our large cities.

New York City for example, with its six million people, consumes approximately two million quarts of milk a day. Practically no milk is produced in the city; most of it comes much more than fifty miles. This vast quantity must be

collected in six different states from a radius of four hundred miles — a great milk "shed" including over forty thousand dairies, which pour their milk toward the great metropolis through the collecting stations, or creameries, and then by train or truck on to the enormous distributing plants which finally deliver the milk to the consumer. There is a wonderful and complicated history of a modern, scientific industry in the production and handling of each quart of milk that reaches our doorstep in the great modern city. The dairy industry has had to be resourceful to provide such vast quantities of sweet milk, every bottle of which must be safe every day of the year; and applied microbiology is the key to the whole problem.

Milk is a very perishable food. Ordinary fresh milk left in a warm place sours in a surprisingly short time. Bacteria are, of course, the agents that cause milk to sour or to spoil. Milk is not only a nearly ideal food for man, but it also proves to be a most favorable medium for the growth of many bacteria, including even some of the pathogenic forms. We frequently use fat-free milk in the laboratory as a culture medium. The average composition of cow's milk is given in the accompanying table.

TABLE XII. AVERAGE PERCENTAGE COMPOSITION OF COW'S MILK AND HUMAN MILK

	Cow's	HUMAN
Fat	4.0	3.3
Sugar	5.0	7.0
Proteids	3.3	1.1
Salts	0.7	.3
Water	87.0	88.3

As we shall see, milk invariably contains relatively large numbers of bacteria, and is menaced at every step in its handling by the possible addition of bacteria to it. It is only by means of sterilization, clean production and handling, continuous chilling to temperatures unfavorable for the devel-

opment of bacteria, rapid transportation, and, finally, the scientific wholesale pasteurization of milk, that the public needs and the public health requirements are satisfied and that a sweet and wholesome food is provided.

Occurrence of bacteria in milk. Raw milk is practically never a sterile product. As secreted and retained in the alveolar spaces of the mammary glands of a healthy cow it is a sterile fluid, but as it passes through the ducts to the milk pail it picks up bacteria. These organisms that commonly reside in the external ducts of the gland and in the cistern, or expansion of the teat duct at its upper end, are harmless and may be considered as normal in milk. Most of these bacteria are washed out by the first few streams of milk drawn (the foremilk), while toward the end of milking there are very few. This fact is frequently taken advantage of in the production of the highest grades of milk, where the foremilk is kept separate and fed to stock. Under ordinary conditions the number of bacteria added to milk from this source is an insignificant fraction of the total number found.

Occasionally an animal is found which, though perfectly healthy, continuously yields large numbers of bacteria in milk. Such an animal could not be tolerated in a herd producing certified milk, and would seriously affect the count of bacteria in any good milk.

As the milk leaves the teats it is exposed immediately to contamination. The air of the dairy barn is far from sterile, and some bacteria are washed into, or settle in, the milk pail. Flakes or hairs from the body of the animal, and bacteria from the hands of the milker, may be contributed. Careless coughing or sneezing projects tiny droplets containing mouth bacteria which may get into the pail. The pail itself may not have been thoroughly sterilized between milkings.

It is evident that the milking process itself is a strategic point, and that not only may large numbers of bacteria be added to the milk at this point, but disease organisms, either from the animal or from the milkman, may be contributed

at this time. If both the cow and the man are free from disease, the danger of infection is practically eliminated; and to gain this end, legislation and education should provide that only healthy men shall milk healthy cows. This ideal condition, of course, is difficult of attainment. The intelligence and the conscience of the dairyman play a large rôle here, as in all other matters pertaining to good dairying.

To exclude bacteria from milk the air of the barn must be kept as free from dust as possible. Sweeping, or feeding hay, just before milking is obviously bad practice. A damp, clean cloth passed over the udder, belly, and flanks of the cow at the

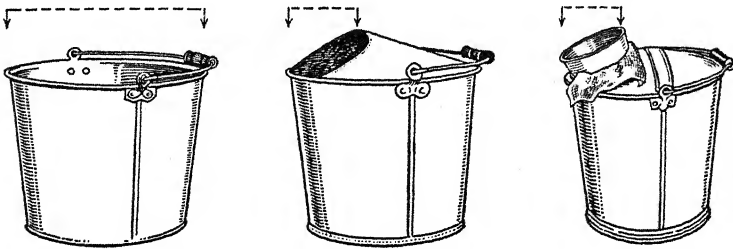


FIG. 42. The old, open type of milk pail, and two of the new, covered types

time of milking will tend to cause dirt and hairs to adhere to the body during milking. Washing the hands thoroughly before milking, and milking with dry hands, will reduce the contamination from this source.

The most important single factor, and the one perhaps most easily remedied but most commonly neglected, is the milk pail. Two things are essential: reducing the size of the top, or opening, and sterilizing. After sterilizing, the pail should be inverted, so as to drain, and should be left in this position in a clean, dry room until the next milking. The small-top pail reduces the area exposed to settling dirt of all kinds. The sterilization prevents the growth of bacteria on the surfaces and in the crevices of the pail between milkings. How important this factor is, is seen from the table on the following page.

TABLE XIII. AVERAGE COUNT OF BACTERIA IN MILK DRAWN INTO STEAMED AND UNSTEAMED UTENSILS IN THREE DIFFERENT BARN¹

	BACTERIA PER CUBIC CENTIMETER	
	Sterilized Utensils	Unsterilized Utensils
Barn I	4,865	311,000
Barn II	3,157	326,880
Barn III	12,400	218,930

All milk utensils should be thoroughly rinsed and washed before being subjected to the sterilizing process. The first rinsing in cold or lukewarm water removes a certain amount of milk, cream, and foreign matter and reduces the burden of washing. Washing is done with warm water to which is added an alkali or soda-ash washing powder (not soap), scrubbing being done with brushes which will reach all parts of the receptacles. After a final rinsing in clean water the utensils are exposed for five or ten minutes to live steam, which will certainly destroy all disease bacteria and most other forms of microbes. It does not sterilize in the technical sense of destroying all living forms present.

In large dairies and creameries special equipment may be used for generating steam and for holding the utensils. Large shipping cans should be sterilized at central stations before they are returned to the dairy. The small dairyman may have the local plumber construct a tight galvanized-iron sterilizer, which may be placed over any source of heat available. The utensils are placed in the box and inverted, and steam is generated from water not over half or three quarters of an inch deep, placed in the bottom.

Where there is only a single pail to sterilize, after washing and rinsing, the pail itself may be partly filled with scalding water and placed on the stove, covered, and boiled for a few minutes. A few bottles may be exposed to boiling water in a large kettle.

¹ Adapted from *Bulletin No. 204*, University of Illinois Agricultural Experiment Station.

Utensils should not be wiped out, but should be drained, and stored in a clean, dry place.

The milking machine practically eliminates the danger of contamination from the air and the milkman; but its expense, and the difficulty of keeping all the parts clean and sterile, have prevented its more general adoption. Theoretically it is ideal; but in practice, from the standpoint of sanitation at

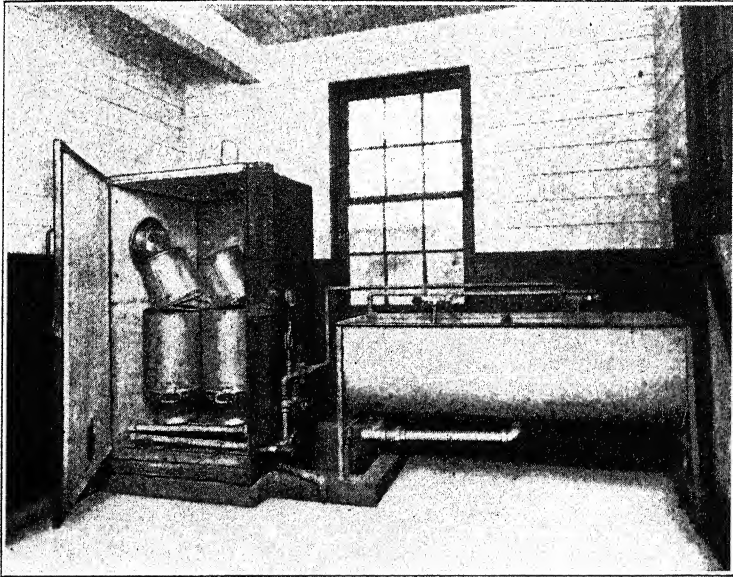


FIG. 43. A simple wash sink and sterilizing cabinet for milk utensils¹

Note compartments in the sink; one for washing, the other for rinsing

least, it has not met all the requirements. For large dairies scientifically handled, with ample facilities for sterilizing, it has much to recommend it.

After leaving the dairy barn, milk may be passed through a variety of receptacles or machines. It may be strained through cheesecloth into the large shipping cans; poured from these into large mixing vats; sent through clarifiers and thence to bottling machines; or it may go first to pasteurizing vats, then

¹ *Farmers' Bulletin No. 1473*, United States Department of Agriculture.

over cooling devices, and then to the bottling machine and into bottles. There are but two guiding principles from the standpoint of bacteriology in all these manipulations: (1) cleanliness and sterilization, between operations, of all parts of the machinery with which the milk comes in contact, and (2) the maintenance of a continuous low temperature in the milk.

The importance of maintaining a low temperature in the milk has been constantly stressed. Ice, or abundant cold spring water at the dairy, is next in importance to the cow herself. Milk is such an admirable medium for the growth of bacteria that they must be continuously inhibited by this means or the milk will sour. All precautions taken to exclude bacteria will be futile unless the growth of the few that inevitably enter is prevented by the use of low temperature. How important this consideration is, is seen in Table XIV.

TABLE XIV. RELATION OF TEMPERATURE TO MULTIPLICATION OF BACTERIA IN MILK¹

Original number of bacteria in milk	5,000 per cubic centimeter
After 24 hours at 42° F.	2,400 per cubic centimeter
After 24 hours at 50° F.	7,000 per cubic centimeter
After 24 hours at 65° F.	280,000 per cubic centimeter
After 24 hours at 95° F.	12,500,000 per cubic centimeter

The varieties of bacteria in milk are determined by those that gain access to it in production and handling. The relative numbers of different kinds of bacteria found in the final product may depend upon their ability to multiply in the milk at the temperature at which it is held.

Numbers of bacteria found in milk. The keeping qualities and safety of milk are determined largely by the numbers and kinds of microorganisms present. Changes in the consistency or the taste of milk are accompanied by the presence of enormous numbers of bacteria multiplying in it. Diseases are spread by this food only when pathogenic bacteria have gained access to it during production or handling. All things considered, the bacterial content of milk when it reaches the consumer is the best index we have of its sanitary quality.

¹ P. G. Heineman, Milk.

The numbers of bacteria found in market milk vary enormously in different samples, but the numbers are always correlated with the complete sanitary history of the milk. No more striking example of sanitary progress could be found than by comparing the bacteriology of milk in one of our large cities today and twenty years ago. In Washington, D.C., in the summer of 1906 the general milk supply contained an average of 22,134,000 bacteria per cubic centimeter, and 11,270,000 per cubic centimeter in 1907. The counts in the same city today rarely run over 30,000 per cubic centimeter. In Boston, in 1908, of milk samples taken from the wagon 27.85 per cent had over 500,000 per cubic centimeter, and 15 per cent had over 1,000,000 per cubic centimeter. Only 41.02 per cent had less than 50,000 per cubic centimeter. In 1925 only 9.5 per cent of samples from the wagon, and 14 per cent of store samples, contained over 100,000 bacteria per cubic centimeter, and in 1933, 86.38 per cent of milk samples taken from delivery wagons contained less than 25,000, and only 1.40 per cent contained above 50,000 bacteria per cubic centimeter. A similar improvement is shown in many of our large cities. These lower counts are due to better dairying, to better refrigeration during transportation, and, most of all, to pasteurization. Unquestionably the quality and bacterial content of raw milk is greatly improved; but the art and practice of pasteurization is the most important single factor, as, efficiently performed, in the average market milk it destroys 98 per cent and over of all organisms present. A high quality of pasteurized milk should contain not more than 25,000 bacteria per cubic centimeter when delivered to the consumer.

It is entirely possible to produce milk under special dairy conditions, in a scientific manner, that will contain 10,000 or fewer bacteria. In 1893 the first Medical Milk Commission was established in New Jersey, to set up legal requirements and to supervise the production of a high-grade raw milk, which was called *certified milk*. The idea proved so successful that by 1920 there were over eighty such commissions in the United States. While certified milk has not proved to be

a solution of the milk problem, it has pioneered in the idea of grading milk according to its sanitary quality. Certified milk as produced today must comply in the methods of production and in its quality with very rigid standards prescribed by the American Medical Milk Commission. These include requirements that the milk shall come from tuberculosis-free herds and that there shall be routine examination of the cows by veterinaries, and of the milk handlers by physicians; and they specify the details as to the construction of the milk plant and equipment. Finally, certified milk shall contain not more than 10,000 bacteria to the cubic centimeter. It is possible to produce milk with this low count year in and year out, summer and winter.

Grading milk. Until rather recently¹ there has been no recognition of the fact that there are differences in the value of different milks, — differences in their nutritive value and especially in their sanitary quality. It costs more to produce clean milk than dirty milk, and the dairyman is entitled to some additional compensation for a high-quality milk. Today the general principle of official grading of milk according to its sanitary quality is well recognized. Grading ordinances should rapidly improve the sanitary quality of milk, increase the practice of pasteurization, yield a more adequate return to the milk-producer, and increase milk consumption by giving the public increased confidence in the safety and value of this food.

Most grading ordinances provide for a high-grade raw milk and for one or more grades of pasteurized milk. Small cities or towns may have to admit more than one grade of raw milk to meet the practical situation. The bacterial count is the most important and tangible test for grading.

Grade A raw milk should be of the highest quality that it is practicable to produce. It should come from herds free from tuberculosis, from dairies of good construction, and it should be carefully handled. Different cities require counts of not over 10,000 to 50,000 bacteria per cubic centimeter.

¹A commission on standards was first appointed by the New York Milk Committee in 1911.

Grade A pasteurized milk should be produced from healthy cattle and under cleanly conditions, and should be pasteurized according to official regulations. Most ordinances require heating the milk to 142°–145° F. for thirty minutes, followed by chilling to 50° F. or lower. This temperature and time assure the destruction of all pathogenic bacteria, and the great majority of all organisms. Massachusetts specifies not over 10,000 bacteria per cubic centimeter for Grade A milk when delivered.

Grade B pasteurized milk is a satisfactory milk, produced under average conditions and rendered safe by pasteurization. The bulk of market milk will ordinarily fall into this category.

Grade C pasteurized milk is sometimes provided for. This would include any low-grade milk not included in the other classifications, and is not to be used except in cooking.

Other names and other systems of grading may be used, but the principle is the same. Unofficial classifications of milk adopted by a private dealer, as "baby milk," "pure milk," etc., are often misleading and give the consumer no special security.

The kinds of microorganisms in milk. It has been noted that the changes undergone by milk are the result of microbic fermentation. The kinds of saprophytic bacteria occurring in milk may be conveniently grouped according to their most characteristic fermentative activity. In addition, several kinds of pathogenic bacteria may gain access to milk either from the cow or from human beings.

The following is a convenient scheme for the classification of microorganisms in milk:

1. Saprophytic microorganisms:

- a. Bacteria producing acid, usually coagulating the casein.
- b. Bacteria producing acid and also gas.
- c. Inert bacteria, producing no demonstrable changes in milk.
- d. Alkali-forming bacteria, which increase the alkalinity in milk.
- e. Peptonizing bacteria, which digest the proteins in milk.
- f. Other bacteria, which affect the color or consistency of milk.
- g. Microorganisms other than bacteria: yeasts, torulæ, and molds.

2. Pathogenic microorganisms:

- a. From the animal.
- b. From human beings.

The most common change in milk is souring, or becoming acid and clotting. The *lactic acid bacteria* include the most common organisms found in milk, and because of their ubiquity may be considered as more or less normal. The group which can produce lactic acid from lactose without gas production has no characteristic morphology. They are usually short rods, or cocci. The form most frequently associated with natural souring of milk is a coccus, or very short rod, usually occurring in pairs or short chains called *Streptococcus lactis*. This organism is nonpathogenic. It causes a prompt clotting of milk, and the acid produced tends to inhibit many other forms of bacteria that may be present. It is not readily grown on culture media, giving only sparse growth, but in milk grows vigorously through a considerable range of temperature.

The *Lactobacillus* group is of special interest because it includes the famous "bacillus of long life" (*Lacto. bulgaricus*) discovered by Metchnikoff. This organism and *Lact. acidophilus* are the cultures commonly used in preparing the commercial sour milks often advised for medicinal purposes. While Metchnikoff's original theory was based upon the idea that his organism might become seeded in the intestine, and, flourishing there, prevent intestinal putrefaction caused by other forms, it is now generally conceded that the cultures do not grow well in the intestine. However, the acid milk diet is often salutary. The lactic acid group of bacteria generally play a rôle in the manufacture of butter and the ripening of certain cheeses.

The *acid-gas-formers* found in milk belong to the intestinal group and are distinctly undesirable. Their presence means the admission of manurial or sewage matter to the milk, with the accompanying risk of pathogenic organisms. While the colon group is not so assiduously sought in the bacteriological tests of milk as is the case in the analysis of water, their discovery, nevertheless, has the same sanitary significance. They are usually concealed because of their small relative number.

The *inert* group, including the thermophilic bacteria, and the *alkali-formers* play an inconspicuous rôle in milk. Their presence in vast numbers may be overlooked because the milk undergoes no demonstrable change. Excessive numbers of such bacteria, however, indicate improper handling somewhere along the line. The thermophilic bacteria which grow at unusually high temperatures may even multiply during the pasteurization period and are occasionally very troublesome.

The *peptonizers* represent the *putrefactive* group. They are undesirable in milk, and usually gain access to it with dirt and filth. Ordinarily they do not play an important part in dairying, as they are masked and inhibited by the vigorously growing lactic-acid-formers. However, they include many of the resistant spore-formers and might be expected to be among those surviving pasteurization. Even in pasteurized milk they scarcely keep pace with the growth of the few remaining lactic-acid-formers; so that pasteurized milk may sour, but ordinarily not with the same clean, pleasing alteration which occurs in raw milk. If the sour milk is kept, the acid will be slowly neutralized by bacterial action, and a digestion of the casein, due to the proteolytic enzymes, will occur. Many of the putrefactive bacteria also produce a clotting enzyme which coagulates the milk though it is still "sweet."

Occasionally *chromogenic* bacteria develop in milk to such an extent that they impart to it an unusual color. This group of organisms has no special significance in dairying.

Milk may become slimy, ropy, or stringy owing to increased viscosity produced by certain capsulated bacteria. Such milk is relished in some countries, and the preparation of curd for the making of Edam cheese involves the culture of an organism which renders it viscous.

Yeasts, *torulæ*, and the spores of *molds* are usually present in milk. Their numbers are insignificant as compared with the bacteria, and they usually develop only after milk has become old. Some of them, which may be artificially added, play a major rôle in the making of fermented-milk beverages used in certain foreign countries and in the ripening of cheeses.

Pathogenic bacteria. Disease-producing bacteria gain access to milk either from the cow or from human sources. All the sanitary precautions so far considered aim primarily at obtaining a safe milk, and incidentally a cleaner food that will keep longer.

The most important disease spread through milk is bovine tuberculosis. While not identical with the human type of tubercle bacillus, the organism causing the disease in cattle may infect children, chiefly those under five years of age. The organism gains access to the milk during milking, chiefly with barnyard filth or flakes that drop into the pail from surfaces that the infected animal may have licked. Rarely the udder itself may be infected and contribute large numbers of organisms directly. The virulent organisms have frequently been isolated from raw milk by animal inoculation. This type of tubercle bacillus has been found in infected organs of babies and little children who died from tuberculosis. No one will attempt to refute that it would be criminal purposely to feed babies disease germs, yet it has been difficult to arouse public interest to prohibit the distribution of infected milk. The remedy lies in freeing our herds of tuberculous cattle and in pasteurization. Infected animals may be discovered by the tuberculin test and removed from the herds. The test is specific, and reliable in from 95 to 98 per cent of cases, as shown by post-mortem examination of animals. It is the chief reliance of the government in eradicating tuberculosis from dairy cattle.

Other diseases are occasionally communicated from cows. Epidemic septic sore throat, milk-borne, is caused by a hæmolytic streptococcus, which may be accidentally planted in the udder from a milkman suffering from sore throat and is then, after an interval, discharged into the milk. The cow appears to play the part of intermediary, the infection being initially of human origin.

Udders infected with any pyogenic organisms may potentially infect milk. Apparently this is the origin of many digestive upsets in infants.

As long ago as 1887 Bruce discovered that a disease supposed to be indigenous to the island of Malta and near-by Mediterranean countries was spread by goats' milk and was caused by a small coccobacillus. Within the last fifteen years it has been demonstrated that there are closely related bacteria which occur in swine and cattle, causing infectious abortion in them and capable of being transmitted to man through cows' milk. These bacteria belong to the genus *Brucella* and have been found to be quite widespread in dairy cattle in this and in other countries. They cause undulant fever in man, and there is reason to believe that this disease is far more common than the reported cases would indicate. In 1930 at least 1385 cases occurred in the United States.

Human diseases, milk-borne, gain access to milk through direct contamination with nose and mouth secretions or intestinal wastes, due to unclean habits or carelessness. Typhoid, diphtheria, and scarlet fever are the most common epidemic diseases traced to milk. Septic sore throat may also be contributed to milk directly from human throats. Other diseases, such as human tuberculosis, may be spread through this medium, but proof of this is lacking. Clean personal habits by all who handle milk and the exclusion of any and all persons with symptoms of disease from contact with milk, coupled with pasteurization, would prevent these milk-borne diseases.

Pasteurization of milk. The object of the milk industry is to provide a fresh, wholesome, clean food that can in no way spread disease to the consumer. Milk, being an animal secretion that runs the gantlet of so much potential contact with human beings, is in constant danger of infection in spite of all precautions. Likewise, the average raw milk inevitably has more living microbes present than it is pleasant to contemplate drinking in a glassful. Therefore any measure that renders milk perfectly safe and enhances its keeping quality without injuring its palatability and food value is a most important adjunct to the milk problem. These requirements are fulfilled by the practice of pasteurization performed according to official specifications and subject to official inspection.

The question of the best temperature for pasteurization has received a great deal of scientific study, and the question of the ideal temperature is not answered even today. The object is to find the lowest temperature that will certainly kill 100 per cent of the most resistant pathogens found in milk in the time allowed. In earlier practice very high temperatures (195° F.) for a few seconds were used, but there were legitimate objections to this. The temperatures and time adopted today leave a fair margin of safety and yet do not materially impair the commercial value (cream line), the flavor, or the nutritive value, but do kill the more resistant tubercle bacillus and all the other (less resistant) pathogens found in milk. Reference to Fig. 35 shows at what temperatures different organisms are killed during pasteurization.

Pasteurization as practiced today is of two kinds: (1) the "holder" method and (2) the "rapid heating" method. The former method is the most common, and ordinarily consists in heating milk to a temperature of between 142° F. and 144° F. for a period of thirty minutes, followed by prompt chilling to 50° F. or lower. The "rapid heating" pasteurization is in a way a return to an earlier practice, which was found unreliable, of using a higher temperature for a very short time. More rigid control over the temperature and the rate of flow of milk through the heating chamber by automatic electric regulators has resulted in the general sanction of this procedure by health authorities. The usual requirements are that the milk shall be heated to a temperature of 160° F. for sixteen seconds and then promptly chilled to 50° F. or lower. Pasteurized milk — all commercial milk, for that matter — should be bottled and capped by machinery to avoid unnecessary risk of contamination of the milk or the bottle from the fingers.

Pasteurization may be performed in the home. At best this is not reliable; and if pasteurized milk is not obtainable and the milk must be used for babies or very young children, it should be boiled. Boiling, as everyone knows, materially alters the appearance, flavor, and nutritive properties of milk. It does render the milk safe, however, and the

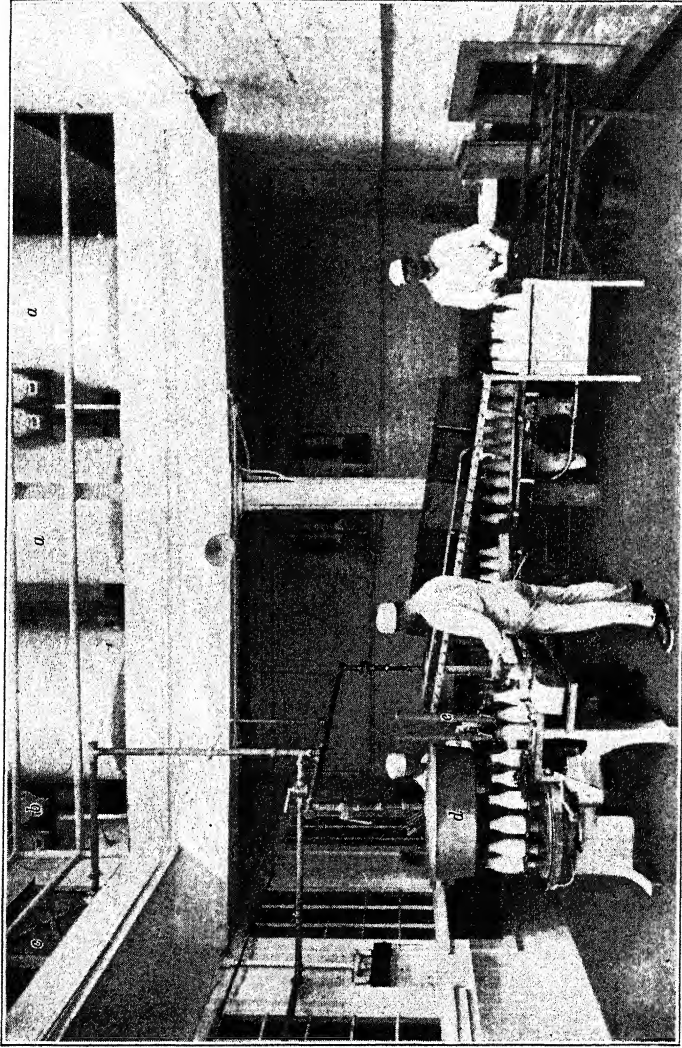


FIG. 44. A city milk plant

a, holding tank for heated milk; *b*, temperature recorder; *c*, bottling machine; *d*, milk cooler; *e*, capping machine

vitamins and other food elements impaired are easily substituted in the diet. Boiled milk has been used for generations by many European peoples, and it is commonly recommended for infant-feeding by pediatricians.

Pasteurization was first used, it will be recalled, to prevent abnormal fermentations in wines and beer. It was first applied to milk by the Danes to improve the flavor of butter and to prevent tuberculosis in calves. It is extraordinary that it did not at once occur to man that he could likewise prevent diseases in babies by a similar practice; but until about 1910 pasteurization met with the most bitter opposition from the medical profession as well as from the layman. Since man heats practically all his animal foods before eating, it would seem perfectly natural to heat milk; in fact, many Europeans have for generations boiled their milk before using it, without untoward results.

The objections have been numerous. The more important ones, with a counterstatement, are given succinctly below:

1. Pasteurization impairs the nutritive value of milk, especially the vitamins.

1. No demonstrable chemical changes are caused by pasteurizing temperatures. Vitamins are only slightly impaired, especially water-soluble C. They are important in milk only to babies on an exclusive milk diet, and correct feeding requires some fruit or vegetable juice which will provide the deficiency in any case. There may be rare exceptions with delicate children.

2. Pasteurization encourages careless dairying and handling.

2. Emphatically, pasteurization is not a substitute for cleanliness. It only makes reasonable safety approximately perfect.

3. Pasteurization destroys the cream line.

3. Careful studies show that, properly performed, the effect on the cream line is negligible.

4. Pasteurization may be improperly performed and give a false idea of security.

4. Pasteurization should never be improperly performed. The procedure should be officially defined, preferably by state ordinance, and should be officially inspected from time to time.

5. Defects in operation and equipment of pasteurizing plants, such as dead ends at outlets, leakage of valves, and foaming, prevent some of the milk from reaching the proper temperature.

6. Pasteurized milk does not undergo an attractive souring, but becomes bitter, or "rots."

5. Recent studies have revealed such defects; but by the introduction of special devices, checked by official inspection, these mechanical imperfections can be eliminated. They by no means outweigh the protection from milk-borne diseases provided by pasteurization.

6. It must be admitted that pasteurized milk does not always undergo as clean and attractive souring as raw milk. However, not all lactic acid bacteria are destroyed. Sour milk for cooking may be procured by once getting a "starter" from a good raw milk which is allowed to sour, and then adding it to pasteurized milk and carrying it along from bottle to bottle. This objection does not outweigh the advantages.

Practically all opposition has now disappeared. The practice is becoming universal in our large cities; and many cities and towns and some states require the pasteurization of all milk except certified or other highest-quality milks which are too expensive for the average consumer. Milk-borne epidemics, and infection with the bovine tubercle bacillus, have rapidly declined with the increase in pasteurization of milk.

TABLE XV. DEATHS FROM NONPULMONARY TUBERCULOSIS IN NEW YORK CITY. RATES PER 100,000 POPULATION¹

(Compulsory pasteurization was established in 1914)

YEAR	RATE	YEAR	RATE	YEAR	RATE	YEAR	RATE
1910	29	1914	27	1918	24	1922	13
1911	30	1915	27	1919	20	1923	12
1912	28	1916	23	1920	17	1924	14
1913	28	1917	24	1921	14	1925	12

The testing of milk. Chemical tests are performed to determine the potential nutritive value and to guard against adulteration or the addition of antiseptics. The tests for butter fat and total solids are the most important.

¹From *New York City Weekly Bulletin*, April 21, 1926.

The sanitary tests are performed either to detect the presence of dirt or — by far the most important bacteriological tests — to determine the numbers and sometimes the kinds of bacteria present.

Tests for dirt are called sediment tests. The most common method is to filter a quart of milk through clean cotton disks of uniform diameter (usually one inch). Milk properly clarified, or strained, will ordinarily give only traces of dirt. The amount collected may be compared with an arbitrary standard (standard gauges may be obtained), and the relative amounts of sediment in milk recorded as "clean," "slightly dirty," "dirty," etc. Such tests are easily comprehended and are impressive to show to the dairyman.

Another practical and impressive test that may be performed without any elaborate equipment, and that gives a general indication of the bacterial content of milk, is the *reductase test*. The presence and the amount of reducing enzyme are dependent upon the number of bacteria present. Methylene blue solution is added to 10 cc. of milk, which is kept in a water bath at approximately 37° C., and the time for the disappearance of the color is noted. The following table is a rough index of the number of bacteria present in milk decolorized in different periods.

TABLE XVI. REDUCTASE TEST

TIME NECESSARY FOR DECOLORIZING	NUMBER OF BACTERIA PER CUBIC CENTIMETER
20 minutes or less	20,000,000 or over
2 hours to 20 minutes	4,000,000 to 20,000,000
5½ hours to 2 hours	500,000 to 4,000,000
More than 5 hours	Less than 500,000

Bacteriological analyses are made either by direct microscopic examination of the milk or by culture methods. The microscopic count, although not accurate, has the advantage of giving quick results, tells something of the morphological types of bacteria present, and reveals the bacteria present, dead or alive, in clumps or single. The culture method demonstrates those that will grow on culture media, and differentiates

their physiological characters, such as acid or gas formation. It is probably much more reliable for milks of low bacterial content, and comparative counts check more closely. Many laboratories make a quick survey by the microscopic method, and subject suspicious samples to more complete analysis on culture media.

Numbers rather than kinds of bacteria are the sanitary guide in milk analysis. The counts run so high that it is exceedingly difficult to isolate contaminants from any given source, such as sewage, and pathogens can rarely be identified except by animal inoculation, as with tuberculosis. Long-chained streptococci revealed under the microscope in association with abnormal numbers of body cells (pus) are indicative of danger; and if, when cultured on blood agar, they dissolve, or hæmolyze, the red blood cells, further investigations should be undertaken at once to locate the trouble. In the production of certified milk the mere presence of long-chained streptococci is often considered a danger signal which should lead promptly to the search for their origin and to the elimination from the herd of the cow contributing them.

The routine plate counts of milk are made by plating the diluted samples on standard agar media and incubating at 37.5° C. for forty-eight hours. The significance of the counts has already been considered. The efficiency of pasteurization may be controlled by counts made on the raw milk and on pasteurized samples respectively.

The complete procedure for the bacteriological examination of milk according to the Standard Methods of the American Public Health Association will be found in Appendix C.

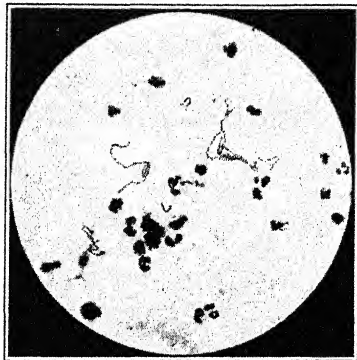


FIG. 45. Microscopic appearance of milk from a cow suffering from mastitis

CHAPTER XIV

MILK PRODUCTS

In addition to whole liquid milk there are several foods prepared or manufactured from milk and cream. Microorganisms play a dominant part in the preparation of several of these foods, as butter and cheese, and are always important from the sanitary consideration and because of the menace of deterioration and spoilage.

Ice cream. Ice cream has become one of the most popular foods in America. Its consumption has increased enormously in the last few years; it is estimated that the annual consumption is well over a gallon per capita. As a commercial food, ice cream has a highly variable content. A very poor ice cream may contain from 6 to 8 per cent or less of butter fat; a very rich mixture, from 20 to 25 per cent. The other ingredients include milk or condensed milk, some *stabilizer* such as gelatin, eggs, or gum tragacanth, to insure the texture and prevent the formation of crystals, and various flavoring extracts. The bacteria found in ice cream are derived chiefly from the cream and milk, but inferior stabilizers may be important sources also.

The number of bacteria found in commercial ice cream is often astonishingly high. Cream contains many more bacteria than whole milk, owing to the fact that in rising, or upon mechanical separation, the fat globules apparently enmesh the bacteria and thereby concentrate them in the cream. Investigations of ice cream in Philadelphia in 1905-1906 showed the average count to be 17,800,000 per cubic centimeter; and in Boston, in 1906-1907, the average count was 23,000,000 per cubic centimeter. With the general improvement of the quality of milk we should expect marked improvement in ice cream; and such is the case, although today the cheaper ice

creams are often loaded with bacteria. In Boston, in 1926, only 8.5 per cent of the eight hundred and eight samples officially examined contained over 1,000,000 bacteria per cubic centimeter. The following table shows the results of fifteen samples collected at random in Boston from high-grade and cheaper drug stores and restaurants. The counts were made on standard agar after forty-eight hours' incubation at 37° C. At least three plates were made of each dilution. It was interesting to note that the richer, higher-grade samples showed more bacteria than the poorer grades. This is doubtless explained by the higher butter-fat content in the rich ice creams; for the cream would contribute the greatest proportion of bacteria, unless it was pasteurized.

TABLE XVII. BACTERIAL CONTENT OF FIFTEEN SAMPLES OF ICE CREAM COLLECTED IN BOSTON, NOVEMBER, 1926

(Analyses by Isabel Linscott)

	NUMBER OF SAMPLES	BACT. COLI PRESENT IN 1 CUBIC CENTIMETER
Under 50,000	4	2
Between 50,000 and 100,000	3	2
Between 100,000 and 500,000	5	1
Between 500,000 and 1,000,000	2	1
Over 1,000,000	1	0

The apparent number of bacteria in ice cream increases appreciably during the freezing process owing to the breaking up of clumps. Upon storage there is at first a sharp decline, followed for a few days by an increase, and again a rapid decline, which decreases in rate till a nearly stable number is reached. A recent study shows that an increase may appear after a month or more of storage. As long as the water present is not actually crystallized, this is, of course, possible. From the chemical and commercial standpoint, ice cream does not deteriorate during storage, and is sometimes held in refrigerators at 0° C. or slightly below for months. There seems to be no legitimate objection to this practice, and it makes

possible the conservation of cream produced in excess of demand at certain seasons of the year.

Cream may be infected with the same bacteria that gain access to milk, and so, in theory at least, may spread disease. Experience shows ice cream to be of minor importance in this regard. Instances of "ice-cream poisoning" — probably acute infections with intestinal organisms that have multiplied enormously prior to freezing — are recorded. It is reasonable to suppose also that the bovine tubercle bacillus may be spread by this food. Clean, wholesome dairy products and other ingredients should be used. The pasteurization of cream to be used in ice cream has the same sanitary advantages that it has with fluid milk.

Butter. Butter is manufactured from the fat of milk obtained by skimming or mechanical separation, and is further concentrated by the churning process, which causes the tiny fat globules to cohere in a mass.

Practically always the cream is *ripened*, or allowed to undergo a mild souring, before being churned. This ripening is brought about by bacteria and is responsible in large measure for the flavor of the butter. Ripening may occur as the result of spontaneous souring, but such a haphazard method has been largely supplanted by the use of starters or of pure cultures. This makes for uniformity and brings under control the flavor, which largely determines the commercial value of the product.

Starters are grown in skim milk. When the milk has curdled, but before free whey is exuded, it contains the largest number of active organisms, and is then added to the cream and thoroughly mixed. The rapid development of acid inhibits the growth of undesirable organisms and prevents the development of disagreeable flavors. The amount of acid, and probably other by-products of fermentation, determines the flavor and the intensity of flavor of the butter.

Pure-culture starters consist of single or mixed cultures that are isolated and carried along in laboratories. They are used in the same way as natural starters.

The greatest control over butter-making should result from the use of pure cultures added to pasteurized cream. This practice would also control any danger from the spread of disease through butter, particularly tuberculosis, the bacillus of which has been frequently isolated from butter. For practical reasons pasteurization of cream preparatory to making butter has not been universally adopted. Cities and states having pasteurizing ordinances for milk should consider the necessity of protecting the public health, by the same means, from infection spread by butter.

Butter keeps well, partly because of its low water content and the high concentration of salt dissolved in this water, and also because butter fat is fairly resistant to fermentation. Butter can be stored at temperatures below the freezing point without impairment. The deterioration of butter and the development of rancidity is little understood, but it is doubtless due in part to the slow growth and action of bacteria and their enzymes.

Cheese. We have noted the ready decomposition of milk due to microorganisms. When this decomposition is controlled we get various pleasing and palatable foods in the form of different kinds of cheeses. The manufacture of cheese is almost wholly dependent upon the action of microbic enzymes, and the preservation of the food properties depends upon the inhibition of one group of organisms by another. The variety of textures and flavors that we get in different cheeses is astonishing when we consider that the raw product from which they are made is the same, the one variable being the character of fermentation that it undergoes.

Cheeses are variously classified. We may group them as acid-curd and rennet-curd cheeses, according to whether the initial clotting of the milk is caused by an acid fermentation or by the addition of rennet.

The acid-curd cheeses include the fresh, soft cheeses that must ordinarily be consumed fairly soon. While not of great commercial importance, from the sanitary viewpoint they are far more likely to spread disease than those cheeses that

undergo a prolonged ripening and storage. Studies in Washington, D. C., by Schroeder and Brett, showed that as high as $14\frac{3}{4}$ per cent of market samples made from unpasteurized milk contained tubercle bacilli. Typhoid epidemics have been traced to this type of cheese. It should be made from pasteurized products only. In Washington, following the enforcement of pasteurization, of the 122 samples tested not one contained living tubercle bacilli.

The commercially important cheeses are chiefly made by the use of rennet. These, in turn, may develop into hard or soft cheeses, the consistency depending upon the amount of whey left in the curd or upon the ripening process which the curd undergoes.

The rather tough rennet curd is gradually digested by proteolytic bacterial enzymes, and the characteristic flavors and consistencies are produced. The American cream cheeses, factory cheese, Swiss cheese, and others remain hard and firm. Holes, or *eyes*, of irregular size and distribution develop, owing to gas fermentation, and determine the texture of the cheese. This is especially striking in Swiss cheese, which exhibits large, rather uniform holes, evenly distributed except near the surface. In these hard cheeses the lactic acid group of bacteria are usually numerically dominant. The proteolytic enzyme pepsin, contained in the rennin, is activated by the increasing acidity in the curd and plays a part in cheese-ripening. There are always small numbers of proteolytic bacteria present also, which must play a rôle, though how important a one is not understood. Putrefaction is inhibited by the persisting high acid content in the cheese.

Abnormal changes in cheeses are due to the growth of undesirable organisms. The colon-aërogenes group cause excessive development of gas and may affect the flavor, while other bacteria cause bitter tastes or unfavorable odors or colors, and spots. These unfavorable growths may be largely controlled by sanitation, pasteurization, and the use of artificial starters; in other words, by the use of scientific methods which take advantage of our knowledge of modern microbiology.

In the soft rennet-curd cheese a very different kind of fermentation occurs, owing to the higher water content. The ripening process is very complicated, and in only a few cases do we understand the cycle of growth and the organisms involved. The Camembert cheese is ripened chiefly by a mold, *Penicillium camembertii*. This cheese must be made in small units, as the mold growth occurs on the surface. At first the curd is rapidly acidified; this process is followed by the growth of neutralizing organisms, chiefly *Oidium lactis*, which, in turn, renders conditions favorable to the growth of *Penicillium*. This cheese can be manufactured now in almost any part of the world by controlling the culture conditions and artificially introducing the appropriate mold.

In Roquefort cheese the principal ripening agent is likewise a *Penicillium*. Limburger and other highly flavored and odored cheeses are very evidently ripened by organisms of the proteolytic types.

Condensed, evaporated, and dry milk. Besides these milk products, whole milk may be treated and modified to enhance its keeping qualities. Condensed milk is prepared by reducing the water content, adding cane sugar, and sealing it in cans. Evaporated milk likewise is concentrated, but no sugar is added. In dry milk practically all the water is removed, and the solids are reduced to a fine white powder. The principles involved are wholly devised to prevent the growth of micro-organisms that would spoil milk, and they will be considered in more detail in the chapter on food preservation.

CHAPTER XV

FOOD PRESERVATION: DRYING; PRESERVATIVES

Food in sufficient amount and variety is the basic necessity for life and health. The growth and distribution of populations are dependent upon food production, and, consciously or unconsciously, food supply is behind most of our general social and political tendencies, such as migrations, wars, and tariffs. The enormous increase in human populations during the last century and a half has been made possible by the increased food supply due to the opening up of vast new agricultural lands in North and South America, Australia, and other parts of the world, the introduction of farm machinery, the development of rapid transportation, and the postponement of spoilage by the application of scientific methods of preservation. While the production of food has increased, the relative number of persons engaged in agriculture has decreased, as shown in the accompanying table.

TABLE XVIII. THE RELATIVE INCREASE IN URBAN AND RURAL POPULATIONS IN THE UNITED STATES¹

	1930	1920	1910	1900
Urban	68,954,000	54,304,000	42,166,000	30,380,000
Ten-year increase	14,654,000	12,138,000	11,280,000	
Rural	53,820,000	51,406,000	49,806,000	45,614,000
Ten-year increase	2,314,000	1,600,000	4,192,000	

Benjamin Franklin, Thomas Malthus, and others long ago pointed out the tendency that population has to outstrip food supply. Malthus's "Essay on the Principle of Population," published in 1798, first presented the issues squarely and backed them up with statistical evidence. He stated that

¹ From United States Census Report, 1930.

"unchecked, population tends to double in twenty-five years," and that the pressure of populations upon the means of subsistence explains in large part social unrest and human misery. His thesis has had a powerful influence upon social and biological philosophy since that time.

Although from time to time we hear sinister warnings of the "reappearance of the ghost of Malthus," today we find the people of the Western world struggling with the problem of an overproduction of food. Greater yields per acre, due to intensive cropping and more scientific treatment of the soil, better conservation of the food produced, and lowered birth rates, coupled with the restriction of immigration in America, especially have retarded the growth of population on the one hand and increased the food supply on the other.

The rule of season. Most foods are produced seasonally. This is apparent enough with truck-garden crops which are obtainable in a fresh state during only a few weeks of the year. It is equally true of cereal crops and fruits. Animal foods also are to a large extent seasonal. Migrating fish, such as salmon and herring, are harvested for only a limited time. Domestic animals and their products have a more even distribution; but with practically all of them there is, nevertheless, an excess of production over consumption at certain seasons. Thus, for eggs, March, April, and May are the big months; for butter, June, July, and August; for poultry, November, December, and January; for beef, September, October, and November.

Civilized man has long realized the necessity of hoarding food, during these seasons of nature's bounty, against the time of scant production. To be able to do this successfully with the more perishable foods has required a knowledge of the causes of deterioration and of methods for controlling these causes. The application of such methods tends to equalize distribution and therefore prices. It is both economical and promotive of the public health.

Causes of changes in foods. Sound, fresh foods are subject to change from two causes: natural enzymes within the

food itself, and fermentation due to the invasion of microorganisms from the outside. The enzymes in fruits are the agents that cause the desirable ripening. They soften the tissues, change the starches to sugars, reduce the amount of acid, and bring about other chemical alterations. Enzymes are also present in all fresh vegetables and in animal food products. While their action may be favorable up to a certain point, it may continue and render food unpalatable or unwholesome. These agents are an important consideration in food preservation.

Bacteria, yeasts, and molds, while commonly absent from the healthy living tissues of animals and plants, are always present on the external surfaces. Upon slaughtering or harvesting, the life processes are suspended, and the *vital resistance* of the tissues no longer operates. The tissues are rapidly invaded by a wide variety of microorganisms; and unless measures to arrest their growth are taken, chemical changes, usually deleterious, rapidly ensue. It has been noted in previous chapters that in some cases these microbic fermentations are fostered. In most foods microbial growth is always undesirable and causes unwholesome changes.

The object of food preservation is to conserve all food in a sound, natural, wholesome condition until it reaches the consumer. This is accomplished by preventing unfavorable changes due to autolytic enzymes and microbial fermentations.

The perishability of a food is determined largely by its composition. Most important is its water content. Water in some amount is present in all foods. In it are dissolved the salts, sugars, and acids present. Cereals, like rice, wheat, or corn, may have as low as from 10 to 15 per cent water content, while leafy vegetables, tomatoes, and fruits may have from 80 to 95 per cent of water.

Foods rich in protein are subject to highly objectionable changes. Practically all raw food products contain some nitrogen. Sugars and starches may be freed from them during manufacture. Animal foods—meat, eggs, milk, and fish—are richest in proteins, and, as we well know, are most perishable.

Foods rich in sugars and starches are subject to acid fermentations unless their concentration is sufficient to exert an antiseptic action. Acids, as in fruits, may tend to destroy microorganisms or at least to limit the variety that can grow in the food.

Fats are resistant to microbial attack, and, not being miscible with water, are less subject to spoilage than most foods.

In general, then, foods are stable in inverse proportion to their water content and according to their chemical composition. Inactivation of bacteria generally follows when the moisture falls below 40 per cent. Cereals and their manufactured products and sugar are easily kept. Eggs, fish, and meat are very perishable.

Guiding principles in food preservation. Most vegetables and fruits reach a stage of maturity, after which their natural enzymes tend to soften them and they become subject to invasion by molds or bacteria. Animal products, such as eggs and milk, are mature and sound when they are produced. With poultry — chicken, turkey, duck — and with lamb, pork, and beef there is a prime condition when the animals should be slaughtered. The first guiding principle is, then, the harvesting of foods when they are in their optimum state as to their keeping qualities and their use as human food.

The second principle is the careful inspection and sorting of the food, to discover any that is unsound or diseased and to eliminate such.

Thirdly, the products should be handled, exposed, and shipped under clean, sanitary conditions.

Fourthly, they should be promptly chilled or otherwise subjected to the treatment that will best preserve them in their natural condition. Obviously there is often a choice of methods, for the same commodity.

Underlying principles of food preservation. Food preservation is an application of the general principles of antiseptics and disinfection — methods of inhibiting or destroying microorganisms in food. Nature's chief method is drying. The seeds of

plants are preserved against the premature activity of their own enzymes, or destruction by microbes, by the natural removal of water. Mature seeds (cereals) need little treatment beyond careful protection from moisture. Any organic food may be dried, or dehydrated by mechanical means, to preserve it.

Cold inhibits microbial growth and enzyme action. Perhaps the application of low temperatures is the most universal of all methods of food preservation. The home ice box and the cold cellar are almost universal adjuncts of the home, while commercial refrigeration and cold storage are indispensable to the food industry.

The use of "natural" chemical preservatives has the sanction of ancient usage in the salting or smoking of fish and meats by primitive people. Its extension to include antiseptic agents more recently discovered has been slow, and rightly so, as we must be sure that such chemicals have no injurious physiological effects before they are accepted. Oftentimes drying and the action of chemical antiseptics act together; for the extraction of water increases the concentration of some natural preservative present, such as sugar or acids.

Finally, the application of high temperatures to destroy microbes present in food is a most important practice. We have discussed the pasteurization of milk in this regard. Ordinary cooking tends to sterilize foods, and it must be considered as a method of postponing spoilage, as well as a great sanitary measure to protect us from infection by food. The art of canning aims to destroy organisms in food, and to protect it from subsequent contamination by hermetically sealing the container. The exclusion of air was originally supposed to be the chief object, as fermentation was interpreted as being a chemical process activated by contact with the air. Sealing today has a dual importance in excluding organisms and in preventing the growth of aerobic organisms that not infrequently survive the heating process.

Preservation by drying and dehydration. The removal of water from a food tends to preserve it, either because the low

water content itself inhibits the growth of microorganisms, or because of the increase in the concentration of solutes. The method of drying by the use of heat or sunlight may reduce the number of organisms initially present. Molds require less moisture than yeasts or bacteria, and will grow on relatively dry, protected surfaces, the mycelium penetrating the deeper tissues, which may contain moisture. Molds and especially yeasts can withstand high osmotic pressures that completely destroy or suppress most bacteria. Therefore dried fruits, meats, jellies, and sirups are subject to fermentation by molds and yeasts rather than by bacteria.

The method of drying depends partly upon the texture of the food (whether it can be broken into small particles), the effect due to the rapidity of drying, and whether the temperature used alters the flavor and appearance. Natural drying in the sun or by air currents may be used for fruits. Evaporation may be hastened by heat, as with vegetables, or the water may even be distilled off by boiling, as in maple sirup. Fish and meat may be preserved by drying supplemented by the addition of antiseptics, as in smoking or salting. The salt abstracts water from the tissue, and, going into concentrated solution on the surface, suppresses surface growths. Water may also be removed by centrifugation or by pressing, as is done with some fruits and vegetables.

In the initial preparation of many foods water is excluded, a process which results in a high concentration of sugars or salt. Various vegetable oils, such as olive and cottonseed, or animal fats, as butter and lard, are nearly dry. Butter may contain a considerable percentage of water; but if salted, the salt is dissolved in the water, giving a sufficient concentration to be inhibitory.

Where the drying depends upon mechanically controlled processes, it may be spoken of as *dehydration*. These methods are most nearly perfected for the treatment of liquid or semi-solid foods, such as milk and eggs. Such products may be dried by being injected in a fine spray into a warm chamber from which the air has been partly exhausted. The milk or egg

particles are dried almost instantaneously, and settle as a fine powder containing as little as 2 per cent of moisture. Another method is to spread the liquid in a thin film on a heated roller, the dried product being continuously scraped off in flakes.

Dehydrated and dried foods keep indefinitely and retain their nutritive properties, and when moistened they recover their natural appearance and flavor to a surprising extent.

The bacteria in the raw food are partly destroyed by the initial processes, and enzyme activities are retarded or completely arrested. If the foods are properly protected the bacteria tend to die off, though contamination may be received from extraneous sources by careless exposure or handling. When foods are moistened or dissolved preparatory to consumption, growth may occur more rapidly than in the fresh products; such preparations should be used promptly, and not made up in such quantities that they will be kept over from one day to the next.

Dried foods must be continuously protected from moisture by being put up in sealed, waterproof packages. There is great economy in shipping and keeping such foods because of their relatively small bulk and because refrigeration is unnecessary. They are a special blessing in the tropics and in exploration.

Preservation of food by chemicals. This practice differs from all other methods of preservation because something is added to the food to enhance its keeping qualities. Chemicals may be added in conjunction with other methods of food preservation; but the sole aim is always to restrict the damaging microbial fermentations by the use of antiseptics that have no harmful physiological effects when consumed in the quantities that would ordinarily be taken with the food.

Preservatives may be arbitrarily divided into *natural* and *artificial*. Those that are natural ingredients of foods or that serve as food themselves, such as sugar, salt, and vinegar, have the sanction of long usage. Others, as sodium benzoate, boric acid, and formaldehyde, may be present in natural foods — benzoic acid in cranberries, for example — but their

use is more recent and more artificial. The use of these preservatives gives an incentive to conceal inferiority in foods; and their legitimate use is restricted to a few thoroughly tested chemicals in special foods where a minimum of preservative may be essential, as in catsup. The Pure Food and Drugs Act, passed by Congress in 1906, carefully regulates this whole practice, with great benefit to the public, which it protects from fraudulent and dangerous practices.

Preservatives may be antiseptic because of their physical action by altering osmotic tension, as with salt and sugar, or by chemical action where there is such a reaction between the organism and the chemical as inhibits growth. Pickling in brine and the making of jellies and jams are examples of the former process. In the manufacture of pickles from cucumbers and other vegetables, the produce is covered with a brine of from 15 to 16 per cent. Brine of this concentration is inhibitory to most microorganisms; but lactic-acid-forming bacteria, and especially *Mycoderma vini* and *torulæ*, grow in a film at the surface, as is revealed when the scum is observed under the microscope. The acid is not appreciably destroyed by the surface growths, and the brine and acid together will preserve the pickles for a year or more.

Jellies and jams made from fruit juices are partially sterilized in the cooking process; and the natural acids in the juices, coupled with the high concentration of sugar — usually about 50 per cent — inhibit all but the most resistant yeasts and molds. The receptacles must be protected from surface contamination by sealed tops, or by paraffin, and they should be kept in a dry, cool place. Jellies are not sterile, and easily ferment if air and the ubiquitous mold spores are admitted to the surface, or if they are kept in too warm a place. Sodium benzoate is often added to commercial jams and jellies, in order further to insure their keeping qualities.

Vinegar is a good food preservative. Five to ten per cent acetic acid has a strong antiseptic action. Lactic acid, produced by fermentation due to the *Lactobacillus* group in sauerkraut and related products, serves as a preservative.

Most spices, or their extracts, are antiseptic. Cloves are particularly good. Mincemeat, a highly perishable food, is preserved with vinegar and spices.

These are only samples of the use of preservatives. Salt fish, corned beef, cured ham, olives, condensed milk, and a wide variety of other foods are treated in one way or another by the addition of chemicals to enhance their keeping qualities and, incidentally, in some, to impart pleasing flavors. The use of preservatives is limited, and in the commercial field should be permitted only where the methods of refrigeration, canning, or drying are inadequate or unsatisfactory.

CHAPTER XVI

FOOD PRESERVATION: REFRIGERATION AND CANNING

Preservation of food by the use of low temperatures. The use of cold in order partially or completely to arrest enzymic changes and microbial growth is the most natural method of preserving food. Nothing is added to or detracted from the product, as in the use of preservatives and drying, nor is the chemical composition of the food altered, as is the case in canning. The practice of refrigeration in the home and local market is old, but its general commercial use and its use during transportation are of quite recent origin. It is of tremendous practical importance and benefit, as it extends the range of the food market to the ends of the earth for the community with sufficient purchasing power. The growth of our great urban centers is practically dependent upon refrigeration for an adequate food supply.

Cold acts as an antiseptic rather than as a disinfectant. The growth and activities of microbes are inhibited; the organisms are not necessarily killed. Nevertheless, most bacteria die off slowly when held at a temperature below that which admits of their multiplication. There is no question but that some psychrophilic bacteria will multiply and cause slow decomposition at temperatures of 0°C. , or even below freezing, if solutes are present which will depress the freezing, or crystallizing, point of the water. As we have seen, the freezing of pure water immediately kills the majority of organisms of whatever species, but in foods containing albuminous matter and fats the solidifying is much less destructive. All pathogenic bacteria apt to be found in food, excepting possibly the tubercle bacillus, rapidly die off at low temperatures. The poisons of bacterial origin are not affected by cold, and inferior food is not enhanced by storage. Animal parasites,

notably the *Trichinella spiralis*, or "measly pork worm," are destroyed by storage below freezing temperatures in three weeks or more.

The economic advantages of refrigeration and cold storage are evident. Not only is a more abundant and more varied food supply available throughout the year, but by conserving great quantities of food produced remotely from the places of greatest consumption, and because of the excessive seasonal production of certain foods, refrigeration and cold storage tend to lower and to equalize prices. The storage-warehouse man does not hoard for exorbitant prices: he conserves for the time of need. The rule of season and the principles underlying the cost and risks of storage are immutable laws which do not allow unlimited storage either as to amount or as to time. That there is no incentive to hold food longer than from one season to the next is seen from the following table:

TABLE XIX. PER CENT OF TOTAL RECEIPTS DELIVERED FROM COLD STORAGE AFTER THREE, SEVEN, AND TEN MONTHS, 1909-1910

MONTHS	BEEF	MUTTON	POULTRY	BUTTER	EGGS
Three	71.2	28.8	75.7	40.2	14.3
Seven	99.0	99.3	96.1	88.4	75.8
Ten	99.7	99.5	98.9	97.8	99.9

Guiding principles in refrigeration and cold storage. The principal factors in the use of low temperatures for food preservation are

1. To secure sound, mature, healthy products, as free as possible from microorganisms.
2. To keep the foods in clean, well-ventilated rooms with a relative humidity of from 65 to 75 per cent.
3. To maintain the lowest temperature necessary in order best to preserve the particular product.
4. To keep in storage only long enough to meet the law of demand and supply.
5. To handle and thaw the food (if frozen) in a clean, sanitary manner.

6. To market promptly.
7. To label cold-storage foods as such.

Refrigeration in the home. The home refrigerator is a specially insulated chamber, cooled by ice or by the circulation of chilled brine, in which a low temperature may be maintained for keeping perishable food for a few days. Ice boxes usually record temperatures of from 6° to 10° C., while electrically operated refrigerators may register lower temperatures, according to their adjustment. The lower compartment of the ice box is the cooler, and the more perishable foods should be placed here. In operation the essential things are to keep the shelves and sides scrupulously clean, to avoid direct contact of food with the shelves, and to keep the doors closed as much as possible.

The cold cellar is a dry, clean, cool, well-insulated and ventilated room, usually in the cellar, where a constant cool temperature may be maintained, to keep larger stocks of less perishable foods, such as potatoes and apples, as well as canned goods, jellies, and pickles. Sudden changes in temperature are especially dangerous, as water may condense on the surface of foods and foster mold growths. Keep the cold cellar clean, dry, and as cool as possible.

Commercial refrigeration and cold storage. As commercially applied on a large scale the use of low temperatures has become a scientifically controlled process. *Refrigeration* is used to defer spoilage in fresh foods long enough to provide for handling, shipping, and marketing. Natural or artificial ice or circulating brine may be used to chill the food and to maintain a low temperature. Temperatures at or above the freezing point are used. Microbes are not killed outright; but their growth is greatly retarded, and many species gradually die. Certain forms, however, may continue to grow slowly, since free water is available, and will induce chemical changes. Fresh fruit and vegetables, as well as fish, fowl, and meats, are promptly chilled and refrigerated for short holding periods and while in transit, in order to preserve their fresh natural texture and flavor.

Cold storage provides for the storage of goods in specially constructed warehouses for longer periods of time, and preserves the surplus foods to meet future demands. Temperatures either above or below the freezing point may be used, each food being held under the special conditions of cold and humidity found by experiment and experience to be best. Where solid freezing, or crystallizing, of the water in the food can be used, the alterations during the storage period are negligible. Unfrozen products slowly deteriorate, owing to intrinsic and microbic causes. Eggs may be stored either broken out of the shell and frozen solid, or in the shell when they cannot be frozen. They provide a good illustration.

Cold storage of eggs. Eggs when newly laid are usually sterile. When bacteria are present they are usually few in number, more being found in the yolk than in the white. *Bact. coli* are absent. The shell, although porous, affords a natural protection against contamination as long as it is kept clean and dry. Moist dirt or filth smeared on the shell results in a rapid invasion of the egg by bacteria. If moisture condenses on the shell, bacteria accidentally present there will rapidly penetrate it. Although the contents of the fresh egg have mildly antiseptic action, if bacteria gain access and the egg is warm they multiply with enormous rapidity. To keep eggs sound and clean, therefore, prompt collection, clean handling, and rapid chilling are necessary. Eggs in the shell should be kept at temperatures just above freezing (0.5° – 10° C.), under carefully adjusted humidity conditions to prevent evaporation. Even under these conditions eggs gradually deteriorate, owing to internal causes as well as to the growth of bacteria. Water is absorbed by the yolk, the vitelline membrane is weakened, and the yolk and white blend (see Fig. 47). Chemical changes which affect the flavor accompany this physical deterioration, and the product has a much lower commercial value. Nine to twelve months is the maximum period that whole eggs can be kept in a satisfactory state even under the best conditions of storage. Table XX shows the magnitude of cold-storage egg holdings and the

natural practical time limit for storage. Frozen egg holdings, in the same table, show a more uniform distribution.

TABLE XX. COLD-STORAGE HOLDINGS OF CASE EGGS (WHOLE) AND FROZEN EGGS. MONTHLY AVERAGE, 1917-1921 AND 1929-1933¹

MONTHS	CASE EGGS IN THOUSANDS OF CASES (000 omitted)		FROZEN EGGS IN THOUSANDS OF POUNDS (000 omitted)	
	1916-1920	1929-1933	1917-1921	1929-1933
January 1	1,202	1,129	14,586	65,509
February 1	256	372	12,602	57,341
March 1	23	185	10,842	51,161
April 1	248	1,443	9,859	55,368
May 1	2,560	4,544	10,624	72,974
June 1	5,251	7,442	14,288	93,074
July 1	6,630	8,893	17,578	103,383
August 1	6,849	9,120	19,531	105,846
September 1	6,472	8,568	21,188	101,104
October 1	5,645	7,338	20,654	93,769
November 1	4,272	5,172	19,051	84,024
December 1	2,466	2,814	20,269	74,850

Only the best eggs are selected for storage in the shell. The general quality or condition of eggs is determined by "candling," or examination in a dark room by holding the egg against a strong cone of light and looking through it (Figs. 46 and 47). The inferior eggs that are still sound and are a safe food are broken from the shell, thoroughly mixed, and either dried or frozen. The worst eggs are used in tanneries.

Eggs are usually frozen solid in tin containers. They may be safely held at continuous low temperatures for long periods with little or no deterioration. That they may be kept as "entirely sound and safe food material" for a period of years was demonstrated by a dramatic lawsuit in which certain eggs seized by the United States government under the Pure Food and Drugs Act were finally released for sale after two years of litigation, since it could not be proved that they were even then decomposed.

The bacterial content of frozen egg mixture is ordinarily very high. The organisms show an initial increase, followed

¹ Adapted from *Statistical Bulletin No. 48*, United States Department of Agriculture.

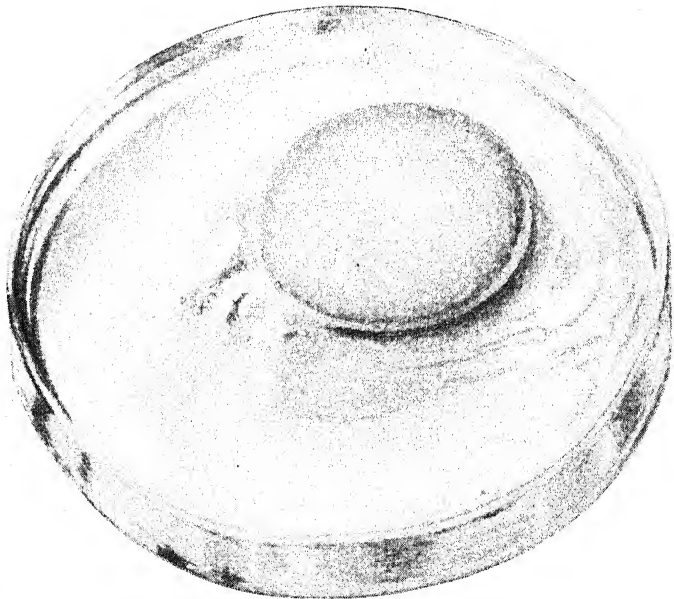
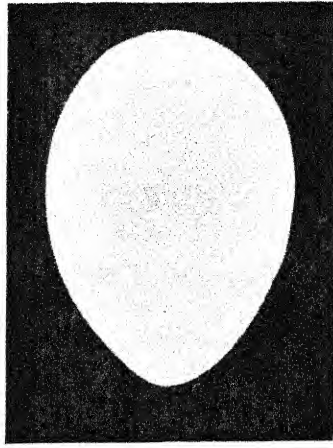


FIG. 46. Fresh egg before the candle and out of the shell¹

¹ Plate XIV in *Bulletin No. 224*, United States Department of Agriculture.

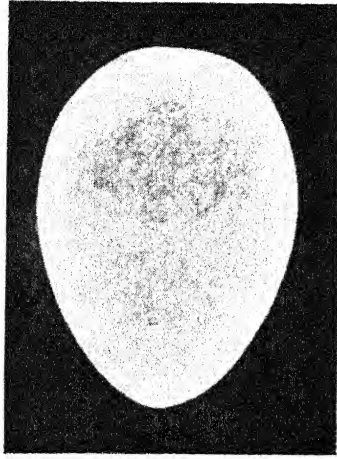


FIG. 47. Egg with soft yolk before the candle and out of the shell¹

¹ Plate XVII in *Bulletin No. 224*, United States Department of Agriculture.

by a reduction over a period of months (see Fig. 48). The eggs when thawed for use are about as good as when frozen, from the viewpoint of their chemical composition, but will undergo rapid bacterial putrefaction and must be used immediately.

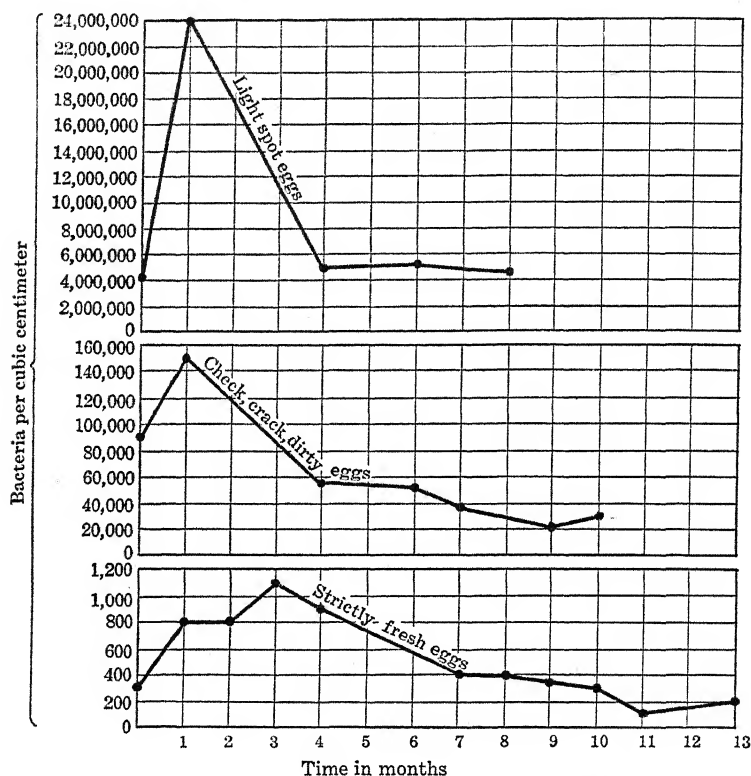


FIG. 48. Graphs showing bacterial content of frozen eggs of known grades, examined at intervals, and averaged by months¹

Poultry. Poultry is a highly perishable commodity, and the problem of getting it from the large sources of production in the Middle West to the centers of consumption thousands of miles away in a fresh, natural condition has been one of the

¹ From chart in *Bulletin No. 158*, p. 30, Bureau of Chemistry, United States Department of Agriculture.

most difficult problems of food-handling. Birds should be starved for twenty-four hours before slaughtering, to reduce the bacterial content of the alimentary tract. They must be killed and bled properly, to prevent discoloration and to enhance the keeping qualities. The animal heat must be rapidly removed by placing in clean, well-ventilated chilling rooms immediately after slaughter.

The study of the relative keeping qualities of drawn (eviscerated) and undrawn poultry has demonstrated in a striking way the advantage of leaving the birds undressed until just before use. The removal of the viscera unavoidably contaminates the body cavity with the intestinal contents and hence with bacteria of putrefaction, which spread rapidly through the tissues; while the intact viscera remaining in the body seal the bacteria in the intestine. The discrepancy in counts as observed by Pennington in eleven shipments is shown in the following table:

TABLE XXI. INCREASE IN NUMBER OF BACTERIA PER GRAM IN THE FLESH OF THE BODY WALL DURING MARKETING, COMPARING FOUR METHODS OF DRESSING FOWLS ¹

STYLE OF DRESSING	PACKING-HOUSE SAMPLE	COMMISSION-HOUSE SAMPLE	FIRST SAMPLE FROM RETAILER	SECOND SAMPLE FROM RETAILER
Undrawn	236	10,790	168,170	1,468,000
Wire-drawn . . .	68,864	82,648	5,481,640	50,759,300
Boston-drawn . .	23,234	341,650	34,258,000	20,309,000
Full-drawn . . .	77,580	9,523,000	1,885,710,000	5,375,270,000

Dry-picked, dry-chilled, dry-packed poultry continuously refrigerated will reach the consumer in the large cities without appreciable deleterious changes. This usually takes three or four weeks. Experimentally it is shown that chemical changes unavoidably set in after about three months.

Poultry may be frozen solid at a low temperature and held at about 15° F. While bacterial growth is completely arrested, slow alterations in texture and flavor are apparent

¹ From *Circular No. 70*, Bureau of Chemistry, United States Department of Agriculture.

after nine months. As with all cold-storage foods, it is of greatest importance that the food be thawed in dry, cool air, and that it be consumed promptly. It would be far better, if acceptable to the consuming public, that fowls be delivered solidly frozen, to be slowly thawed in the home refrigerator.

Meat. Meat may be chilled or solidly frozen for long storage. While most refrigerated meat products probably reach the consumer within a month after slaughter, some meats, particularly beef, may be purposely held for a longer time to *ripen*. The ripening consists of changes in both texture and flavor, brought about by autolytic enzymes and by micro-organisms. Mold growth on the surface is especially prominent, and the progress of ripening is often gauged by the extent of development of molds. Bacteria do not penetrate the meat to any significant depth and do not seem to be concerned in the changes that take place.

Fish. Fish are more seasonal in their production than other animal foods. Certain migratory fish appear for limited periods only, while the inclement winter months make the harvesting of all the principal food fish uncertain and difficult. Fish are subject to rapid bacterial decomposition owing to their high water content. Moreover, they are usually harvested at a distance from warehouses, and special precautions are essential to get this food product to the consumer in a fresh, wholesome state. The practice of freezing and holding fish for even distribution has grown enormously of late years and is an important contribution to human food supplies. In October, 1926, the Bureau of Fisheries reported 70,300,892 pounds of fish held in cold storage, including 12,000,000 pounds of halibut, 11,000,000 pounds of mackerel, and approximately 10,000,000 pounds of all kinds of salmon.

Refrigeration is essential to get fish to market. The modern fishing schooner is always well supplied with cracked ice in which to pack the fresh catch.

Deterioration in fish is evident to the senses. When in good condition the gills are bright red, eyes transparent and bulging, and the flesh is elastic and firm to the pressure of the finger.

Fish may be frozen and held for long periods without deterioration. Fresh, sound, undressed fish are rapidly frozen, and to prevent loss of moisture they are usually glazed by dipping several times in clean water to envelop them in a film of ice. The usual care should be taken with fish removed from storage for marketing.

Preservation of food by canning. Canning is the art and practice of preserving food by destroying all or the great majority of microorganisms present by the use of high temperatures and by storing in hermetically sealed containers. The early investigations of "spontaneous generation" by Spallanzani in 1765, when he boiled meat infusions and sealed the containers to prove that they would not spoil, revealed the essential principles involved. Appert, a Frenchman, thirty years later applied the method to food preservation, using glass containers. Tin cans were first used in 1825.

Canning has become a major method of food preservation only during the last fifty years. Today an amazing assortment of palatable, wholesome foods are offered to the consumer in tin and glass containers, representing in the aggregate an enormous quantity of food. In 1920 over five billion cans of food were commercially produced, besides a vast quantity of home-canned food which is indeterminable in amount. The importance of canning, from the economic and health point of view, hardly needs comment. Scarcely a day passes without our using some food from cans. Modern urban life is well-nigh dependent on canned foods. Modern armies could not subsist in the field without canned food. Exploration has been advanced and made safer because of this boon. Greater abundance and wider variety of food will always promote health.

Of special interest and importance is the fact that vitamins — especially vitamin C, the antiscorbutic accessory contained in abundance in citrus and other fruits and in fresh green vegetables — are destroyed less by canning than by open-kettle cooking.¹ Vitamin activity seems to be independent of

¹ Kohman, Eddy, Carlsson, and Halliday, "Vitamins in Canned Foods," in *Industrial and Engineering Chemistry*, January and March, 1926.

temperature and time of heating, up to a practical point, if oxygen is expelled and excluded during heating.

During the early practices of heating and sealing foods it was supposed that to expel, and subsequently to exclude, air was the essential factor. With the explanation of fermentation as due to bacteria, sterilization seemed the sole requisite. It seems now that many canned foods are not sterile, but the organisms remaining alive are inactive as long as air is excluded; so both factors are fundamental.

The aim in canning should be to heat foods to the temperature and for the time that will assure the keeping of the food, but will alter its texture, color, flavor, and food value as little as possible. Each food product requires special treatment, which will be determined chiefly by (1) its initial microbial content, (2) its viscosity, (3) its acidity, and (4) the presence of natural or artificially added antiseptics.

Processing, or sterilization. The processing, or heating, may be carried out at temperatures of boiling or lower, or at temperatures above the boiling point of water. The term *sterilization*, though commonly used in connection with canning, is frequently inexact in a literal sense, as we have stated that oftentimes aërobic bacteria, commonly spore-formers, or thermophiles, survive the heating.

Pasteurization has a limited use with fluids that have antiseptics present, as beer.

Boiling temperatures are most readily obtained. Practically all living things are killed by *actual boiling in water* for a few minutes. The longer the boiling the more certain its disinfecting action. The outstanding exception to this is the bacterial spore, which is always most troublesome when we try to sterilize anything. It may be destroyed by (1) prolonged boiling, (2) boiling in an acid medium, (3) intermittent boiling, (4) temperatures higher than boiling obtained by processing in steam under pressure, or in salt baths.

The penetration of heat through the entire contents of a receptacle, to reach the temperature of the outside bath or chamber, is a most important consideration. The size and

shape of the container, conduction and convection currents as determined by the consistency of the food, and the agitation of the container determine how long this will take. The nature of the pack — that is, the amount of free liquid filling the interspaces between the pieces of material — obviously affects the rate of heating. The following charts (Figs. 49, 50, 51,

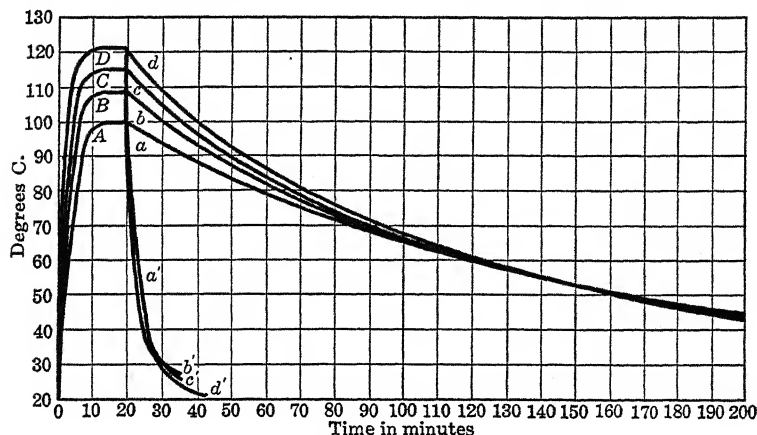


FIG. 49. Curves showing time-temperature relations for string beans in 2 per cent brine, when processed in No. 2 tin cans, and also when cooled in air and in water

Rise in temperature when processed: A, at 100° C.; B, at 109° C.; C, at 116° C.; D, at 121° C. Fall in temperature when cooled: *a'*, from 100° C. in water at 17° C.; *b'*, from 109° C. in water at 17° C.; *c'*, from 116° C. in water at 16½° C.; *d'*, from 121° C. in water at 16½° C.; *a*, from 100° C. in air at 16° to 20° C.; *b*, from 109° C. in air at 19° to 22° C.; *c*, from 116° C. in air at 18° to 22° C.; *d*, from 121° C. in air at 19° to 22° C.¹

52) show strikingly the differences in time required for different foods in uniform-sized tins to reach the temperature of the outside bath.

Fundamental factors in canning. *Sound, fresh foods* only should be used. Canning, or any other method of food preservation, is not to be used to conceal inferiority. Only food that would be acceptable as fresh food should be canned. The method does not improve unwholesome food, but preserves it in as good condition as when placed in the cans.

¹From *Bulletin No. 956*, United States Department of Agriculture.

The *blanching* of fruits and vegetables is largely a cleansing process. Blanching consists of preliminary boiling in an open kettle or exposure to steam for a varying time. This washes off dirt and mucus, loosens the skin, expels the air from the intercellular spaces, and effects an initial destruction of some of the bacteria present.

Sanitation is essential in its broadest application. The food should be clean and free from dirt. The factory or room where

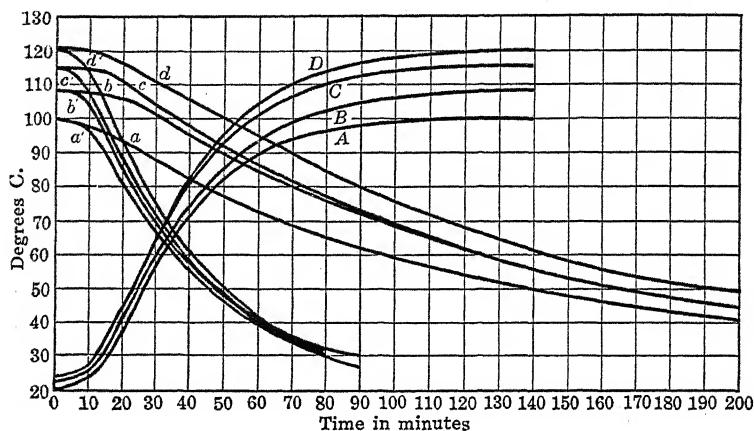


FIG. 50. Curves showing time-temperature relations for sweet corn when processed in No. 2 tin cans and also when cooled in air and in water

Rise in temperature when processed: A, at 100° C.; B, at 109° C.; C, at 116° C.; D, at 121° C. Fall in temperature when cooled: a', from 100° C. in water at 15° C.; b', from 109° C. in water at 22° C.; c', from 116° C. in water at 21° C.; d', from 121° C. in water at 18° C.; a, from 100° C. in air at 22° to 26° C.; b, from 109° C. in air at 25° to 28° C.; c, from 116° C. in air at 25° to 28° C.; d, from 121° C. in air at 25° to 28° C.¹

canning is done should be light, well-ventilated, free from dust, and so constructed and operated that floors, shelves, tables, kettles, and other surfaces are kept in a clean, sanitary condition. This requires an abundant, safe water supply and intelligent supervision.

Suitable *equipment* for handling the food, and final containers that are durable, noncorrosive, and that can be tightly

¹ From *Bulletin No. 956*, United States Department of Agriculture.

sealed, are fundamental. Good glass containers best meet the requirements, except that on account of breakage they are impractical for general commercial use. Cheap glass, containing air bubbles, is dangerous, as the glass splinters during heating, and bits of glass may mix through the food. For general commercial uses tin, lacquered on the inside, is most satisfactory. No appreciable amounts of lead enter into the composition or seal now.

The factors regarding *heating* have already been taken up. The temperatures and times for various vegetable products, either by open processing or under pressure, are noted in the following table:

TABLE XXII. TEMPERATURE TABLE FOR COMMERCIAL CANNING¹

VEGETABLE	BLANCHING	STERILIZING		
		Boiling	Pressure	
			Pounds	Time
Tomatoes	(To loosen skin)	22 min.	20	10 min.
Beets	3-8 min.	90 min.	20	35 min.
Corn	5-10 min.	180 min.	20	35 min.
Beans	2-5 min.	120 min.	20	40 min.
Peas	2-5 min.	120 min.	20	40 min.
Squash	10 min.	90 min.	15	35 min.

Canned goods should be *tested* for spoilage. Certain cans taken at random from each run should be incubated, to see whether any detectable changes occur.

Good cans will always be clean and bright, showing no leaks, and the ends will be tight and drawn in slightly, owing to the partial vacuum in the can caused by the contraction of the air and fluid contents as they cool. "Swells" are cans that are obviously puffed out at both ends owing to gas fermentation due to surviving anaërobic organisms. "Springers" and "flippers" feel loose to pressure by the fingers at one or both ends. "Flat sour" cannot be detected by observation of the can. It is due to acid fermentation caused by non-gas-forming, anaërobic bacteria.

¹ From H. W. Conn and H. J. Conn, *Bacteriology*, p. 214.

When a can is opened there should be suction of air inward. The food should appear and smell natural and attractive. *Do not taste the food if you are suspicious of it.* Either destroy it or *boil it* before testing by the sense of taste. An organism, *Clostridium botulinum*, may grow and produce a *very potent*

poison in canned food. Its poison, or toxin, is destroyed by boiling.

All canned foods should be *stored* in a dry, cool atmosphere. Any residual organisms that survive processing but do not develop during the testing period may still be encouraged to grow slowly if kept in a warm room. Cool storage will reduce such development and may completely prevent the germination of spores.

Canned foods should be used as

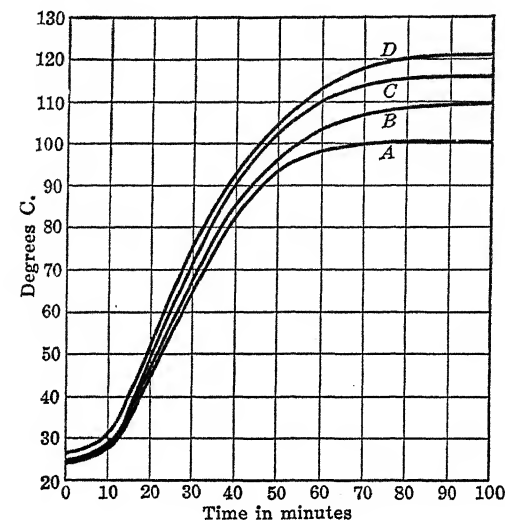


FIG. 51. Curves showing time-temperature relations for pumpkin when processed in pint glass jars at different temperatures

Readings at intervals of five minutes. Rise in temperature when processed: A, at 100° C.; B, at 109° C.; C, at 116° C.; D, at 121° C.¹

soon as possible after opening. There is no special danger in leaving a portion of the food in the can if it is chilled and is not kept until spoilage sets in. Canned food is cooked food, and will deteriorate more rapidly than fresh products.

Bacteria in canned foods. A wide variety of organisms may be found in spoiled canned foods, including bacteria, molds, and occasional yeasts. Living bacteria are also found in unspoiled cans in about 25 per cent of samples examined.

¹ From *Bulletin No. 956*, United States Department of Agriculture.

Aërobic spore-forming bacteria are practically the only forms found in unspoilied cans. The most prevalent species is *B. mesentericus*, followed by *B. subtilis* and *B. vulgatus*. It is evident that the exclusion of the air supply is what prevents this group from multiplying and spoiling the food.

Yeasts, many of which are facultative, and anaërobic spore-forming bacteria, including *Clos. welchii*, usually cause a swelling. Yeasts are bothersome in canned fruits and in condensed milk. Anaërobic bacteria find a wide variety of foods to their liking. Canned sardines may swell owing to the growth of members of the colon group. The intestines are not entirely removed before the fish are packed; so there is a heavy initial seeding of the can with these organisms.

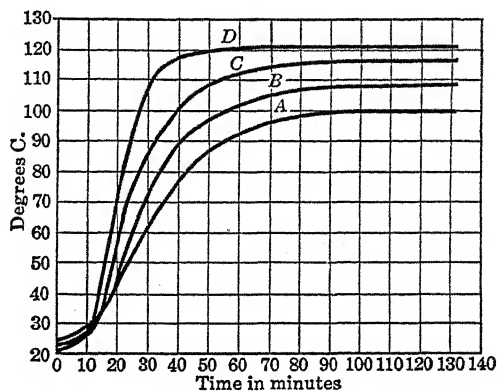


FIG. 52. Curves showing time-temperature relations for tomatoes when processed in No. 2 tin cans at different temperatures

Readings at intervals of five minutes. Rise in temperature when processed: A, at 100° C.; B, at 109° C.; C, at 116° C.; D, at 121° C.¹

Certain little-understood thermophilic bacteria play an important part in flat sour. Two groups have recently been recovered from a variety of canned foods which cause typical flat sour upon reinoculation into corn, peas, beans, spinach, and other vegetables. Since this group does not grow below 40° C., the importance of cool storage is reinforced.

Home canning. The general principles already discussed in commercial canning are applicable to food preservation by this means in the home. Canning in the home is not usually profitable unless there is a surplus domestic production of

¹ From *Bulletin No. 956*, United States Department of Agriculture.

raw fruits or vegetables so that the foods do not enter in as a direct cost item. On the other hand, there is often considerable pride and satisfaction, apart from the monetary aspect, in preparing and preserving clean, wholesome foods for winter use, to take the place, occasionally, of the commercial product of which the origin and handling are unknown. Home canning has become well-nigh universal in the homes of women who do some of their own cooking and who are favorably located for procuring fresh raw foods.

Methods of heating. The most convenient and the usual method of applying heat for sterilization in home canning is in the open water bath. Boiling, except at relatively high altitudes, will destroy most of the microorganisms in acid fruits and vegetables in from five to thirty minutes. The boiling point (212° F. at sea level) is depressed about 2° F. for every thousand feet of increase in altitude, so that at two thousand or three thousand feet it becomes an important factor.

For open-water-bath canning, any covered receptacle sufficiently large to hold a convenient number of jars may be used. The wash boiler serves the purpose well. A rack, or wire basket which will fit the boiler, to support the jars, is essential to prevent bumping and breaking.

A single short heating is sufficient to assure the keeping of most fruits and of a few vegetables like tomatoes and pickled beets. Nonacid foods, and those in which the consistency inhibits the rapid penetration and circulation of the heat throughout the food, are much more difficult to sterilize by boiling temperatures. The United States Department of Agriculture recommends the canning of these foods only by means of the pressure cooker. There is, admittedly, some uncertainty of results and some very slight risk of botulism poisoning in canning corn, string beans, spinach, and other foods of this nature by any means except under pressure; yet botulism is a remote risk compared with other hazards that we take daily — crossing a city street, for example.¹ With

¹ From 1899 to 1922 there was a total of 345 cases, with 213 deaths, from botulism in the United States. Over 35,000 are killed by automobiles each year.

good, intelligent canning technique the keeping of such foods is usually so satisfactory that we are constrained to mention the alternative of longer heating or intermittent heating at boiling temperatures.

It has been stressed that it is absolutely necessary to heat the *entire contents* of the container to the *boiling point*, and that the consistency of the food, the free water content, the tightness of the pack, and the size of the jar are all important practical considerations in this regard. With other than acid foods it is wisest to use jars not exceeding quart capacity.

For such foods as string beans, lima beans, and peas, where the water is free, admitting circulation and the rapid penetration of the heat, three hours' continuous heating after the outer bath begins to boil is recommended. With semisolid foods, as corn or squash, heat penetration is slower, and four or more hours is safer.

Intermittent boiling (discontinuous heating) on three successive days aims to kill all bacteria that are in the vegetative state at each heating. During the intervals between heating, the jars are kept at a moderate temperature, on the theory that the spores will germinate between heatings. While fifteen to thirty minutes actually at boiling temperature will certainly kill all the vegetative cells, it is desirable to extend the period well beyond this time to allow for thorough heat penetration, and also to complete the desirable time for cooking the vegetables. At the first heating the food is already near the boiling temperature, owing to the preliminary cooking or parboiling. During the interval between the second and the third processing the foods have, of course, cooled and will take somewhat longer to come to the boiling temperature. Ordinarily an hour or longer, *after active boiling has commenced* in the container, should be allowed.

Pressure cooking is the most reliable method of canning. Such cookers are similar in construction to the autoclave figured and described elsewhere (see Fig. 21). When the inner chamber is filled with live steam, the outlet is closed and the pressure rises. The temperature increases with the pressure,

and is read on the gauge (see Fig. 22). The time and temperature necessary for processing a few foods are shown in the accompanying table:

TABLE XXIII. TABLE TO SHOW TIME AND TEMPERATURE FOR CANNING A FEW FOODS¹

(All foods are to be packed hot)

PRODUCT	TIME, PRESSURE, AND TEMPERATURE FOR PROCESSING	
	Quart Jars	Pint Jars
String beans	40 min., at 10 lb. pressure, 240° F.	35 min., at 10 lb. pressure, 240° F.
Corn	80 min., at 15 lb. pressure, 250° F.	75 min., at 15 lb. pressure, 250° F.
Lima beans	60 min., at 10 lb. pressure, 240° F.	55 min., at 10 lb. pressure, 240° F.
Peas (green)	50 min., at 10 lb. pressure, 240° F.	40 min., at 10 lb. pressure, 240° F.
Spinach	90 min., at 10 lb. pressure, 240° F.	85 min., at 10 lb. pressure, 240° F.

The container. Glass jars of pint and quart capacity are usually used in home canning, although tin cans for home use, with the necessary equipment for easy sealing, are available.

The most important single consideration regarding containers is that they shall be made to seal absolutely air-tight. The opening should be wide enough for easy packing, and the edges and angles of the glass jar and cover should be smooth, rounding, and accessible for easy cleaning. (Tin receptacles are, of course, discarded after use.) The top and the outer edge must be free from nicks or cracks that may possibly admit air. It is cheaper to throw away the jar or cover than it is to discard later the food that has fermented because of improper sealing.

The rubber rings are important, as they complete the seal. *New*, good-quality rings that are elastic and do not crack when sharply doubled over, should be selected.

¹ Adapted from *Farmers' Bulletin No. 1471*, United States Department of Agriculture.

Packing. Food may be packed into the jars in three general ways :

1. It may be cooked in the open kettle, and packed hot into sterile jars and sealed. This is unsatisfactory, as it is difficult to sterilize the jars and to prevent contamination during the packing manipulation.

2. The food may be blanched or boiled and then dipped into cold water — the *cold-pack* method. Subsequently it is processed in the container. The cold dip does not injure the bacteria present, and it has the disadvantage that the food must be reheated in the container during processing.

3. The food after blanching is *packed hot* into the container and immediately processed. This method has the advantage of commencing the sterilizing process with the entire contents of the jar at a temperature only a little under the boiling point; the food has shrunk and condensed, and the air has been expelled so that it packs tightly. It has obvious advantages over either of the other methods.

Sealing and testing. In the pressure cooker the hot jars are sealed before processing.

In the open process the covers are fitted and the wire clamps adjusted, but not closed, before placing in the holder or basket to be lowered into the boiler. Immediately after the sterilizing process is complete, the covers are clamped (or screwed down) tight, and the jars are tested for leaks by inverting them on a clean towel. Any gas sucking inward or liquid exuding indicates a leak, and the food should be used at once or discarded (or processed over again, with a new ring and cover). It is well to leave the jars at room temperature for a week, and examine each one carefully for spoilage before putting away in the cool preserve closet. The precautions that have been given regarding the careful inspection of canned food before using are even more important with the home product.

CHAPTER XVII

THE MECHANISM OF INFECTION

The relation that microörganisms bear to disease is the most important and at the same time the most interesting application of the science of bacteriology. The influence that the proof of the germ theory of disease has had upon medicine and surgery has been profound. It has revolutionized the theory and practice of medicine, and has changed surgery from a dread method of last resort in desperate cases of sickness to a beneficent, curative, and even preventive procedure fraught with little danger — at least from blood poisoning, or septi-cæmia, which before the days of asepsis was the rule rather than the exception. Preventive medical practices have brought under control some of man's most common and fatal diseases, while modern sanitary science has given a rational means of freeing the environment from the menace of infectious agents. The death rate from all causes in the United States registration area has dropped from 19.8 per thousand in 1880 to 11.4 in 1930; the mean length of life in Massachusetts has increased from 39.77 years in 1855 to 58.32 in 1924, a gain of 18.55 years; typhoid-fever epidemics, which used to appear in our cities as surely as the fall season, have become of rare occurrence; infant-mortality rates have been cut in half. In short, sickness, suffering, and premature death have been greatly lessened, and more abundant life, happiness, and longevity are the fruits of discoveries in bacteriology, all occurring within threescore and ten years.

Contrast the present knowledge of infectious diseases with the ignorance and superstition of the past. The earliest notion of the causation of disease, and one that has persisted in primitive tribes up to the present time, conceived the sick body as possessed by a demon, ghost, or evil spirit that racked

and tortured the person. Treatment consisted in the propitiation of the spirit with sacrifices or promises; or its eviction by magic, or by scaring it away by the beating of tom-toms, or by pommeling or even baking the patient.

Hippocrates had a more natural conception of disease in the theory of the four humors: blood, phlegm, yellow bile, and black bile. The proportion existing between these humors in the body determined its health or disease.

Many curious ideas have been held regarding the causation of disease; but it was not until the renaissance in science, beginning in the seventeenth century with the courageous, independent investigation of such men as Harvey, Sydenham, Hooke, and others, that observation began to supersede the dominating and deadening influence of authority and precedent. The body and its functions began to be studied in a scientific spirit, and the foundations were laid for modern biology. In the early nineteenth century the applications of physics and chemistry to living things made it evident that the body may be looked upon as a mechanism, and that it is subject to wear and tear or that it may be of poor construction. The constitutional or functional nature of certain diseases was firmly established. At about the same time the improved microscope revealed minute parasites as the cause of a disease of silkworms (Bossi, 1837), and a fungus as the cause of a scalp disease in man (Schönlein, 1839). The stage was set for the triumphant work of Pasteur to establish the germ theory of disease as we know it today.

Infection and virulence. *Infection* is an aspect of parasitism. A parasite is an organism that derives a part or all of its sustenance from another living being, called the host. This phenomenon is very common among both plant and animal forms and is subject to all shades and degrees of variation. The parasite may do severe injury to its host, even destroying it, or its effect may be very slight. When the parasite invades the living tissues of the host, and establishes itself more or less permanently therein, it is called an infectious agent, and the process of entering the body constitutes infection.

Virulence is the capacity that an organism has to produce disease. An infectious disease, being essentially a reaction between parasite and host, depends not alone upon the factors inherent in the invading organism but also upon the factors of resistance in the individual attacked. The virulence varies with different species of bacteria and even with different strains of the same culture. Some epidemic outbreaks of scarlet fever, for example, are relatively mild, whereas in other cases it is severe. In the laboratory virulence may be *exalted* or *attenuated* in various ways. Ordinarily the successive passage of a pathogenic organism from one animal to another raises, or exalts, virulence, whereas long-continued growth on artificial culture media weakens or attenuates it. The inoculation of smallpox virus into calves permanently modifies this organism into the harmless and beneficent cowpox virus, which is used in vaccination against smallpox. The virulence of streptococci is increased for mice when inoculated from one of these animals to another, but at the same time virulence is decreased for rabbits.

The recent discovery of dissociation, or mutation, of bacteria has explained the hitherto obscure fluctuation of virulence within a given species or culture. We now know that the presence or absence of a capsule commonly determines the degree of virulence an organism possesses. Capsulated forms, spoken of as "smooth" cultures, are the more virulent. In artificial media they give rise to relatively smooth, glistening colonies on solid media and cause uniform cloudiness in broth. The noncapsulated or "rough" forms, on the other hand, are less virulent, or may be nonvirulent. They appear as drier, rougher colonies and form a floccular growth or sedimentation in liquid media. Mutation, or, as it is usually called, dissociation, from one form to the other may take place within the body of the host (*in vivo*) or in artificial media (*in vitro*). Cultures derived from single cells may give rise to both rough and smooth types.

The capsule probably serves as a protective envelope, shielding the bacteria from the antagonistic and destructive

chemical substances in the blood and cells of the host's body. This enables them to gain a foothold in the body and to grow and invade the body more generally.

As has been stated earlier, the capsule is formed of a highly complex sugar, or polysaccharide. This imparts to the cell highly specific properties, not possessed by noncapsulated bacteria of the same species, which serve to incite the production of special reacting or protective substances when introduced into the animal body. Bacteria that are otherwise very closely related may differ in the chemical nature of the capsule and may be differentiated by certain reactions which will be spoken of later.

Furthermore, this antigenic property, as it is called, is most important to consider in the preparation of bacterial vaccines; for it is apparent that only smooth strains would possess the chemical structure to stimulate the body to its optimum defensive reaction. The severity of different epidemics and the rise and fall of epidemics also may have their explanation in this phenomenon of dissociation.

Whether or not an organism is able to establish itself in the host will be determined by the *portal* by which it enters the body. Most pathogenic microbes have very definite predilections, not only for particular hosts but for specific tissues within the host. The typhoid bacillus cannot invade the body through the skin, but can enter only through a definite area in the small intestine; pyogenic staphylococci normally invade through the skin, and are harmless if swallowed; the whooping-cough bacillus lodges exclusively in a definite area in the trachea; the malaria plasmodium is an exclusive parasite of the red blood corpuscles. The tubercle bacillus, on the contrary, while preferring the respiratory tract as a portal of entry, and the lungs as a preferred site for extensive growth, may also enter the body via the alimentary tract and may infect the glands of the body, the skin, and even the joints and bones.

On the part of the host the factors determining susceptibility or resistance are numerous. Persons who have recovered from a natural infection of measles, diphtheria, or many

other diseases are, ordinarily, unlikely to have the disease a second time. Vaccination against smallpox or typhoid fever renders a person nonsusceptible or immune for a time. Age seems to be an important item: the "children's diseases," like measles, whooping cough, and diphtheria, are not only more prevalent but are much more fatal in the early years of life. The strain of tubercle bacillus infecting cattle rarely attacks persons over the age of five years. Other general factors that temporarily predispose to infection, especially to tuberculosis, are malnutrition, fatigue, alcoholism, and exposure to unfavorable atmospheric conditions, such as excessive heat and cold or humidity and dryness. One disease may predispose to another. Measles and influenza are all too often followed by pneumonia, and the diabetic is prone to invasion by pyogenic bacteria.

How bacteria cause disease. The initial step in an infectious disease is for the organism to gain access to the body by a favorable path and to multiply therein. This ability to grow within the body is the essential difference between parasitic and saprophytic microorganisms. The disease resulting from an infection is due to mechanical injury and to chemical poisons produced by the microbes. The violent, paroxysmal cough in whooping cough is due to the irritation caused by the bacteria in the ciliated surface of the trachea. Tissue destruction occurs in many diseases. In tuberculosis for example, the lung tissue is broken down and may progress until the walls of the blood vessels are weakened and break, causing hemorrhage. The evidences of tissue damage in the scars of the smallpox victim, or following boils or carbuncles, testify to the extensive destruction that has occurred.

The poisons that bacteria produce, however, are more important. These are of two general sorts called exotoxins and endotoxins. The exotoxins are soluble secreted poisons that may by filtration be readily recovered free from the cells that produce them. When introduced into the animal body, they all have the property of inciting the production of a special type of countersubstance, or antibody, called antitoxin. Relatively few of the bacteria produce exotoxins as a

prominent type of poison. Those that do, include diphtheria and tetanus bacilli, the streptococcus of scarlet fever, and certain staphylococci. Also certain forms of food poisoning, notably botulism, caused by the growth of saprophytic bacteria in food, are due to secreted, diffusible exotoxins.

The endotoxins are bound more closely to the bacterial cell or are an integral part of the cell substance, and are liberated only when the cell wall is ruptured or the cell is dissolved. Since recovery from infection requires that the invading bacteria be destroyed, the immunity mechanism itself serves to liberate such toxins. The nature of these intracellular poisons is obscure because they cannot readily be studied as pure products. All pathogenic bacteria possess these poisons, and even the dissolved products from saprophytic bacteria have poisonous properties. This has led to the theory that the endotoxin may not be a preformed chemical entity, but is a toxic radical split off from proteins of the bacterial cell.

Disease-producing microorganisms. Most microorganisms are incapable of producing disease. As a matter of fact, only a very small fraction of the known species are capable of invading the living tissues of either plants or animals. The important types that can infect man do not exceed two score. Generally speaking, parasites have only one specific host and are incapable of causing natural infection in two or more species of plants or animals. There are notable exceptions to this, as with the rabies virus, which can infect dogs, man, horses, cows, and other mammals; or the bacillus causing bubonic plague, which is essentially a disease of rats and other small rodents but which may cause disastrous epidemics in human populations. On the other hand, diseases like scarlet fever or measles never occur naturally except in man, and it is only with the greatest difficulty that they can be transferred experimentally even to such closely related animals as apes or monkeys.

There are representatives of all forms of microorganisms included among the infectious agents of man. Certain minute worms and arachnids, such as the trichina and mites respectively, may burrow into the living tissues.

There are a large number of pathogenic protozoa, including some of the most important infectious agents of man. Malaria is caused by a protozoön belonging to the class *Sporozoa*. This organism (*Plasmodium*) is essentially a parasite of the red blood cells, but has a complicated life cycle which includes a sojourn in the *Anopheles* mosquito, providing a means for transfer from one human host to another. Certain Flagellates cause infections in animals and man. The terrible and fatal African sleeping sickness is caused by *Trypanosoma gambiense*, which is also spread by an insect.

Both *yeasts* and *molds* may be parasitic, although in man they are rare and of relatively little importance. Favus has been mentioned as due to a mold (*Achorion schönleinii*). Ringworm is a communicable disease caused by a fungus (*Trichophyton tonsurans*). Thrush, an infection of infants, causing white patches on the tongue and throat, is due to a yeastlike mold (*Oidium albicans*).

The *filtrable viruses*, or organisms that pass at least one stage of their life cycle in a state which permits them to pass filters so fine that they hold back organisms which may be viewed under the microscope, include a large and important group of pathogenic organisms. Recent work shows that we have been misled in the view we have held toward some of this group, and have mistaken the filter-passing toxins, which may produce symptoms of disease, for the virus itself, or else that in some cases an organism has a filter-passing and a non-filter-passing phase in its life cycle. We may anticipate that further investigation will demonstrate a similar situation with regard to the cause of certain diseases that we now describe as due to "filtrable viruses."

Smallpox is the most important human disease caused by a virus. The organisms of both smallpox and vaccinia will pass through the Chamberland filter. With rabies, or hydrophobia, typical bodies (Negri bodies) (see Frontispiece) are demonstrable in the infected brain cells, but there is also present a filtrable virus. In infantile paralysis, yellow fever, influenza, common colds, typhus fever, and many other diseases the fil-

trate from these bacteria-proof filters contains the infectious agent, as may be demonstrated chiefly by animal inoculation.

The *pathogenic bacteria* are most important and most numerous as the cause of infectious diseases, particularly the Eubacteriales, although members of the higher orders — the Actinomycetales and Spirochætales — include some very important parasitic representatives. Included among the Spirochætes is the *Treponema pallidum*, the causative agent of syphilis, and the spirochætes causing relapsing fevers. The syphilis microbe is a delicate, long, slender, spiraled organism possessing a fine flagellum at one end. The body is quite flexible. It is an exclusive parasite of man and infects other animals with difficulty. It has been grown in the test tube under strict anaërobic conditions. Needless to say, this organism is one of man's worst enemies, not only being spread by contact but also being transmissible from parent to offspring.

In an infection of the mouth known as Vincent's angina a spirochætal organism is found accompanied by a fusiform bacillus (see Frontispiece). The part played by each organism is not clear, but it is an example of a disease of dual or multiple etiology of which there are a number of instances.

Actinomycosis is an infection due to the filamentous branching bacteria. The disease occurs chiefly in cattle, but is met occasionally in man.

Tuberculosis and diphtheria are caused by bacteria belonging to the Mycobacteriaceæ, a family of the "higher" bacteria, which occasionally show branching. These two diseases are discussed more in detail in a succeeding chapter.

The "true" bacteria, Eubacteriales, include most of the pathogenic forms. All morphological types are represented. The Coccaceæ are the chief agents of suppurative processes, such as boils, abscesses, carbuncles, and the like. The streptococcus is the most common ally of other infections, seemingly always present and ready to invade the tissues of the body weakened by other specific microbes. The cocci are also responsible for several primary specific diseases, such as pneumonia, epidemic cerebrospinal meningitis, and gonorrhea.

The Bacteriaceæ are represented among the pathogens by the typhoid, dysentery, influenza, and other rod forms. The typhoid group is discussed more in detail in a succeeding chapter.

The spore-forming rods, the Bacillaceæ, are not commonly infectious. There are two or three important members of this group, however, and their ability to survive indefinitely outside of the body presents a special problem in their control. The organism causing lockjaw, *Clostridium tetani*, is commonly found in the intestine of the horse and becomes widely disseminated in soil. It enters the body with dirt purely by accident; and in special types of wounds—ordinarily deep punctures, or gunshot and powder wounds, which provide anaërobic conditions within the body—it finds favorable conditions for growth. It ordinarily remains localized, its secreted poison spreading through the body and paralyzing certain nerve centers. *Clos. welchii*, the bacillus usually causing gas gangrene, is a common inhabitant of the intestine of man and occurs in infected soil. Infections are rare in civil life but are prominent among soldiers wounded in battle. Anthrax is essentially a disease of cattle and other domestic animals the products of which enter into commerce. Infection with this aërobic, spore-bearing bacillus occurs chiefly among those who handle raw animal products, such as hides, hair, and wool. The extraordinary resistance of the spores enables it to survive all the commercial processes it is put through in manufacture, and to cause infection in the users of the finished product. A number of human cases of anthrax in 1918 were traced to the use of cheap shaving brushes, viable spores being found in the bristles of these brushes.

The only outstanding member of the family Spirillaceæ causing disease is the *Vibrio comma*. This organism usually occurs as a bent rod, a "comma bacillus," rather than as spiraled or corkscrew-shaped. It causes one of the most acute and fatal diseases of man, cholera; but owing to the development of sanitary science, and especially to international and maritime quarantine, cholera has been successfully kept out of northern Europe and America during the present century.

Sources of infection. In order to prevent infectious diseases we must first know where disease germs come from and how they reach successive new hosts — we must know something of the sources and of the modes of infection.

Prior to the establishment of the specific germ nature of disease it was thought that certain disease-producing substances (*materies morbi*) might emanate either from the environment or from sick patients; the former were called miasms, the latter contagiums. The air, or atmosphere, was the chief medium which carried these morbid effluvia. Miasms (Gr., defilement, stain) were supposed to originate in swamps and marshes, and in drains, sewage, and decomposing organic matter of all sorts, and to be wafted to great distances with the air. Dirt, filth, and bad odors, regardless of their origin or nature, were indiscriminately charged with *originating* disease. Sanitary practices must needs be diffuse, general, hit-or-miss, concerned with the abatement of nuisances, plumbing inspection, garbage disposal, and the like, to the neglect of the specific factors which we now recognize as of greatest importance. Malaria was the last stronghold of the "miasmatic" hypothesis, it being stoutly maintained that exposure to "night air" and the proximity of swamps accounted for this disease. When it was proved that a mosquito which bred in swamps, and which bit most freely at night, carried the malaria microbe from person to person, the facts were explained and the miasm theory exploded.

If the disease substance was transferable from one diseased person to another, it was called a contagium. Not being able to recognize the specific nature of this contagium, people supposed it to have extraordinary powers of survival outside of the body and to be capable of wide dissemination through the atmosphere. Attention was therefore focused upon the environment rather than the patient and his infectious body discharges. Quarantine was empirical, and confidence was placed in disinfecting the premises and the atmosphere of the sick room. The distance separating the sick from the well was supposed to be an important consideration. The "pest houses"

for the care of smallpox patients were usually located remote from habitations. In the construction and operation of contagious wards in hospitals the guiding principle was the physical separation of the patients. The technique of nursing and of concurrent disinfection, so as to prevent the escape of germs from the bedside upon the hands of the attendant, or with articles used by the patient, such as eating utensils, clinical thermometers, and bedpans, was not considered important. Even after the discovery of specific microbes as the cause of different diseases the idea persisted that they might live for indefinite periods of time outside the body, and that the air played a major part in their distribution. In those diseases where the cause is still unknown, or only recently discovered, the layman is partial to improbable stories regarding the origin of particular cases, as of scarlet fever or infantile paralysis. In scarlet fever, for example, playthings used by a diseased child months or years previously are brought out for a visiting child, who coincidentally has this disease; or a room formerly occupied by a scarlet-fever patient is repapered, and, scarlet fever being in the neighborhood, a case coincident in time is supposed to be "caught" through the release of these ancient germs from the wall paper. The filth, or pythogenic, theory, the miasm and contagium theories, got so firm a grip upon the lay and medical mind that it has taken a long time to substitute the true facts regarding the origin and spread of disease.

Modern concept of sources and modes. The newer knowledge of sources and modes of infection differs from the old in being definite, specific, and based largely on scientific proof. It has revolutionized the practices of quarantine and sanitary prophylaxis — from the old "shotgun" methods to those of the rifle, where an accurate aim is taken at a definite target. The emphasis has changed from dealing with an infected environment to infected individuals. The contraction of a communicable disease is often a very personal and intimate thing. It can no longer honestly be charged to "acts of God," miasms, and contaminated inanimate objects.

The specific sources of disease microbes are, in their order of importance: (1) human beings, (2) lower animals, and (3) the environment.

Man himself provides the chief and favored habitat for the majority of disease-producing bacteria. It is convenient to discuss the sources of infectious microbes in man according to the site from which they leave the body in search of new hosts. The mouth and nose are of prime importance. Most of the highly communicable (contagious) diseases are spread by the fresh secretions from the mucous membranes of the nose and throat. This material escapes from the body in a great variety of ways. Coughing and sneezing and, to a lesser extent, loud speaking result in a local spray of tiny droplets which are forcibly ejected from the mouth and nose. The air within a few feet is temporarily infected, and the fresh particles are inhaled by another; or the spray settles upon neighboring objects, perhaps food or toys. All sorts of objects are placed in the mouth to moisten them, or for no reason at all. Using the same drinking cup, biting the same apple, swapping pencils which are reciprocally sucked, and an infinite number of other illustrations suggest themselves. Perhaps the fingers are the most flagrant disseminators of germs from the mouth and nose. Chapin exclaims, "Who can doubt that if the salivary glands secreted indigo, the fingers would continually be stained a deep blue; and who can doubt that if the nasal and oral secretions contain the germs of disease, these germs will be almost as constantly found upon the fingers!"

The diseases spread by contact with these fresh respiratory secretions usually infect the tissues of the upper passages of the nose and throat or the lungs. Tonsillitis, pharyngitis, bronchitis, and pneumonia all take their names from the region infected. Whooping cough is obviously a respiratory disease. In such diseases as scarlet fever, measles, cerebrospinal meningitis, and chicken pox the prominent symptoms are skin eruptions or paralysis, and the respiratory link is not so apparent. The earliest symptom of scarlet fever is a sore throat; the first symptoms of measles are well known to be those of a cold and

sore eyes. The microbes of all these diseases escape chiefly from the mouth and nose and not from the skin eruption, although this source is possible with chicken pox.

The alimentary tract constitutes another primary source of infection. The bacteria causing the intestinal infections escape from the body with the alvine wastes and are distributed widely in nature with this filth. The tubercle bacillus, and possibly other organisms that appear in the mouth, may be swallowed and will pass through the intestine alive. However, respiratory diseases are not spread by the intestinal wastes.

The urinogenital tract may harbor disease germs, particularly those of gonorrhea and syphilis. Typhoid and tubercle bacilli may appear in the urine — the latter in the case of tuberculosis of the kidneys.

Infectious microbes may appear in the skin eruptions — especially where there are purulent discharges, as in smallpox — and from boils, discharging ears in scarlet fever, or superficial syphilitic lesions.

Finally, the microorganisms may be true blood parasites, which never come to the mucous or epidermal surfaces. Nature has ingeniously provided for the transfer of these organisms through intermediate insect hosts. Malaria, yellow fever, dengue fever, and typhus are examples of diseases having this origin.

If the human body served as a source of infection only during the active stage of the disease, it would appear as though quarantine, or isolation of the patient, coupled with the concurrent disinfection of the specific infectious wastes from the body, should prove a most effective and reliable preventive method. Unfortunately the problem is not so simple, for individuals may harbor and discharge disease germs while they are unaware of being a source of danger. Bacteria may be eliminated from the body (1) during the incubation period of the disease — that is, the time elapsing between exposure and the appearance of characteristic symptoms; (2) during the active stage of the disease; and (3) following convalescence, for short or long periods of time. A

disease may also be so light as to be missed or unrecognized, or there may be cases in which there are no symptoms at all. The latter cases, together with those who completely recover from a disease and are still in a condition to communicate it, we call *carriers*. The impossibility of successfully controlling diseases by isolation and quarantine of the sick is apparent. The control of susceptible contacts and the discovery of abortive cases and of carriers are likewise necessary and are exceedingly difficult things to accomplish.

Animals serve as sources of disease that infect man. Dogs usually disseminate the rabies virus; cows suffer from a type of tuberculosis that is transmitted to babies through milk; rats are the chief reservoir of the bacilli causing bubonic plague; and various domestic animals suffer from anthrax.

The *environment*, which used to play so transcendent a rôle in the minds of sanitarians fifty years ago, is relegated to a very subordinate position today. Diseases caused by the very resistant spore-forming bacteria, such as tetanus, anthrax, and gas gangrene, may be thought of as environmental, although in all the cases mentioned the natural habitat of the bacteria happens to be the animal body, the bacteria merely sojourning indefinitely in the soil and upon objects or articles of commerce. The "food poisonings," and particularly botulism (which is discussed elsewhere), seem to be caused by organisms that can live saprophytically. Diarrheal diseases in babies are caused by massive numbers of bacteria in milk, regardless of the varieties or sources. With these exceptions and a few other unimportant ones, environment apparently never serves as a primary source of infection.

Resistance of pathogens outside of the body. Microorganisms of disease show a graded resistance to the destructive forces of nature outside of the body. A very rough classification might place them all in four groups:

1. A large majority of pathogenic organisms die in a matter of a few hours when they are discharged from the body and are dried or exposed to the sunlight and to other antiseptic influences. Here would be included microbes that cause the

highly communicable diseases, and that are provided with ready means of rapid transfer from host to host. Such diseases as measles, whooping cough, meningitis, scarlet fever, and gonorrhea will serve as examples.

2. Some bacteria will live from a few days to a few weeks outside of the body, in water or food or upon objects generally. Bacteria causing alimentary diseases belong here, such as the typhoid bacillus and the cholera vibrio, and also some of the respiratory organisms, such as the diphtheria bacillus and the pneumococcus.

3. The tubercle bacillus is one of the most viable non-spore-forming organisms. It will live for weeks or months in a dried state if no special means are taken for its destruction. Certain of the pus-producing cocci likewise seem to be endowed with extraordinary resistance, and this helps to explain why minor cuts are always menaced by these infecting agents.

4. The spore-bearing bacteria have been discussed elsewhere. Those that may cause infection practically always enter the body through breaks in the skin. Their survival outside the body is measured in years.

The longevity of pathogens outside of the body is a most important consideration in its influence upon theories of modes of infection. Those that quickly perish must pass quickly from host to host, while those of greater resistance may use roundabout routes of transfer.

Modes of infection. The path of travel, or method of transfer of microbes in going from host to host, is known as the mode of infection. The transfer may be immediate and direct as regards time and space, or it may be greatly delayed and circuitous. The time element is of supreme importance with some bacteria, while a very specific conveyor, such as an insect, may be essential in another case.

The modes of infection are conveniently classified as (1) by direct contact, (2) by indirect contact, (3) by fomites, (4) by vehicles, and (5) by vectors.

Infection by *direct contact* implies the transfer of fresh, *moist* infectious material from host to host. This includes

not only physical contact of person and person in kissing or handshaking, but also cases of successive contact with food or objects by the person harboring infectious matter and the susceptible person. A common eating utensil, a pencil sucked first by one child and then by another, or mouth spray from a sneeze inhaled by another person a few feet away results in as definite contact with the fresh mouth secretions as would be the case in kissing.

Indirect contact implies a longer time interval between transference of infection. Perhaps for convenience — for there is no arbitrary division between the methods — we can think of the transfer of infectious material that has *dried* upon objects, but in which the microbes are still viable, as constituting indirect contact. Door knobs, telephone receivers, counters, desks, and trolley straps may retain dried, living infectious material for a few hours at least, and this material may be removed upon handling. Dried matter tends to adhere to the objects upon which it is dried, and the part played by indirect contact is slight as compared with direct contact.

Fomites are inanimate objects that may retain virulent microbes for long periods of time — for weeks or even years. From the previous discussion the conclusion is at once drawn that only with a very limited number of diseases caused by very resistant bacteria can fomites play a rôle. Anthrax, spread by hides, wool, or hair, is fomes-borne; staphylococci can apparently survive upon instruments or in dust for protracted periods, waiting for an opportunity to invade through breaks in the skin. The old fetish of fomites is exploded for most communicable diseases.

By *vehicles* of disease we mean food and drink which serve to convey infectious agents from host to host. Water, milk, and shellfish are vehicles of typhoid fever, while other uncooked foods, or cooked foods, such as bakery products, are handled and exposed to potential infectious material from the fingers and mouths of the venders. Flies may likewise infect such foods.

Living agents other than man himself that transfer disease germs are called *vectors*. Insects play the most important part

in this relation. Flies are *passive* vectors, serving accidentally, because of their filthy habits, to convey intestinal bacteria from drains or open privies to food. The microbes are carried on the body surfaces or within the mouth parts or intestine, and are deposited wherever the fly lights and feeds. The *Anopheles* mosquito plays a more dramatic and essential rôle in the spread of malaria. The protozoan in the malaria patient's blood is withdrawn when the mosquito bites, or feeds upon, the patient. A complicated life cycle of the parasite must occur in the body of the mosquito before it can reinfect another human host. The mosquito is a *biological*, or *active*, vector of malaria. There are intermediate relations, as in bubonic plague, where the flea, by actively feeding upon the sick animal, becomes infected, and subsequently inoculates a new host by feeding upon it. There is no essential development of the plague bacilli in the flea. Such an insect may be spoken of as an *inoculating* vector.

It appears from the discussion in this chapter that infection is not a simple phenomenon but is a very complex one. The virulence of the organism, its mode of escape from the host, its ability to survive outside the body, the portal by which it enters another host, and the devices used for reaching these portals of entry, all play a part.

When a pathogenic organism finally reaches its host by the preferred portal, the host on its behalf may be receptive or refractory, susceptible or immune. The next chapter deals with the defensive mechanism of the host.

CHAPTER XVIII

IMMUNITY

It is well known that when a person recovers from an acute infectious disease, such as measles, thereafter he is unlikely to have the same disease a second time. There is an altered state of the body, resulting from the attack of the disease, which endows it with resistance to the specific infection experienced, but not to any other. The person is said to be *immune*.

Any person who has not had measles is very likely to catch it if exposed, or brought into the intimate presence of an incubating or active case of the disease. Such a person is said to be *susceptible* to measles.

Immunity, then, is a condition of nonsusceptibility, a refractory state in which a person has more than ordinary resistance to particular infections, owing to a variety of causes which will be discussed presently. Immunity is not an absolute state of refractoriness; it is a state of resistance well above the average, capable of all degrees of gradation until we arrive at the condition described as susceptible. It is approximately absolute for measles in the recovered measles patient, or for smallpox in the recently vaccinated individual. After a period of seven years, or longer, following smallpox vaccination, the resistance wears off. With some diseases, as colds or influenza, no immunity is acquired — for any appreciable time, at least — although a defensive mechanism must be active to explain recovery from these infections.

Kinds of immunity. For illustration we have discussed the kind of immunity familiar to all. There are several other types, or kinds, of immunity which need to be discussed.

Natural immunity. The different *species* of animals and plants do not all suffer from the same infections. Dogs and cats do

not have measles or typhoid fever, nor does man have distemper or hog cholera. Generally speaking, there is a lack of susceptibility of one species to the infectious diseases to which other species are heir. There are many important exceptions to this, as has been noted. Rabies naturally infects a number of remotely related species, such as dogs, horses, and men; and the bovine tubercle bacillus infects cows, hogs, and men. Animals immune under natural conditions may be rendered susceptible by altering body resistance artificially.

Races may show differences in susceptibility. Tuberculosis is much more fatal among Negroes than among whites, and among the Irish as compared with the Jews. Measles, when introduced into Iceland and the Faroe Islands, showed an appalling fatality among the natives as compared with its usual severity among Europeans. Long racial experience with a disease seems to play an important rôle here.

It is quite apparent that *families* and *individuals* show graded resistance to specific diseases. Not all persons contract typhoid or scarlet fever when exposed, even if they have no known history of these diseases. Susceptibles and immunes in diphtheria and scarlet fever can be accurately determined now by simple tests — the Schick test and the Dick test respectively.

Natural immunity must be due to inheritance. Immunity may also be acquired during gestation, owing to the immune substances passed through the circulation from mother to fetus. In some diseases, what appears to be natural immunity is probably due to the person's having had subclinical or unrecognized attacks of the disease which have rendered him immune.

Acquired immunity. Immunity contracted by artificial or natural processes during the lifetime of the individual is called acquired immunity. Such immunity may be due to the reaction of the body to stimulation by the living infectious agents or their products; or it may be acquired in a wholly passive manner by the reception of immunizing substances produced in another animal body and transferred to the person who will acquire immunity.

Natural acquired immunity results from the experience of disease acquired by accidental exposure and infection. The virulent disease microbes stimulate the body to defend itself; and following convalescence, in most cases, we have a more or less enduring heightened resistance.

The body defenses may be strengthened into *active artificial acquired immunity* in anticipation of the risk of natural infection by inoculating attenuated microbes or their derivatives, in which case the body will respond in a manner similar to that undergone in a natural infection, but with no risk, as the inoculated doses are controlled as to virulence or amount. In smallpox vaccination a permanently weakened virus, carefully purified, is introduced into the skin. A benign, local disease develops, resulting in a general reaction on the part of the body which produces an immunity to smallpox. In typhoid inoculation known numbers of the bacteria, killed by heat, are inoculated into the body in three separate, graded doses about a week apart. The chemical constituents are nearly identical with those that would enter the body during a natural infection, and the reaction is the same. The body acquires immunity. In the production of diphtheria antitoxin the secreted poison, exotoxin, is used to stimulate the production of immunity in the horse. The response is so strong that the blood serum of the horse has a vast excess of the substances (antitoxin) which give immunity in this disease, and these can be recovered for use in combating the disease in human beings. The human body may also be immunized by the injection of suitably neutralized, or weakened, diphtheria toxin.

Passive artificial acquired immunity is produced when immune substances (antibodies), such as diphtheria antitoxin, are inoculated into the body to give it specific protection. The body gives no active response; it is not stimulated to build up its own immunity, but remains a passive recipient of the antibodies produced by another animal. Such immunity is quickly acquired, is at its height immediately after the injection, but wears off gradually as these foreign circulating

substances are destroyed or excreted. In contrast, active immunity is acquired gradually, — sometimes very slowly, as with diphtheria toxin-antitoxin treatment, — but it persists ordinarily for years, or perhaps for the remainder of life. Passive immunity is acquired in an emergency when no other safeguard is certain; active immunity, endowed artificially, is truly preventive and is the most reliable means of controlling those diseases when the method is known. Medical prophylaxis of this sort holds out the greatest promise of definite results, and our knowledge in this field is rapidly growing. Progress is retarded by lack of coöperation on the part of the public, due to a false apprehension as to dangers involved in the practice. It is only by education and by the dissemination of true knowledge of the facts regarding the safety and reliability of these preventive measures that we can gain the full benefit of these remarkable and beneficent discoveries.

The explanation of immunity. The theories to explain the phenomenon of immunity are highly technical, and in an elementary work of this sort a detailed consideration is inappropriate. Some of the simpler explanations may be attempted, however. Parasitism with infectious microbes is an aspect of the struggle for existence. The unrestrained and continued growth of microbes in the body will overpower and destroy it. The body, to survive, therefore, must have a mechanism of defense. On its ability to arrest and eradicate the invaders depends its survival. Hence recovery from disease requires an energetic physiological response to the infecting agents and their poisons. Since the chemical composition of each microbe is slightly different, each one calls forth a definite, specific reaction. Having once acquired the power of such a specific reaction, the physiologic mechanism retains it in an exalted state, and a slight stimulus by the same chemical bodies immediately mobilizes the protective substances, the antibodies.

The body is capable of an infinite number of such specific reactions, not only to bacteria, protozoa, and ultraviruses, or their derivatives, but to all chemical substances of protein

nature and to certain complex carbohydrates. Such substances are called *antigens*. Thus, antibodies are manufactured following the injection of egg albumen, foreign serum, and plant proteins. Some people are hypersensitive to particular foreign substances received into the body either by injection or by contact with the mucosa of the respiratory or alimentary passages. Hay fever and idiosyncrasies respecting food have such a cause. The use of pollen extracts inoculated to build up resistance to hay fever has an explanation similar to that of immunity acquired from infections.

The external defenses of the body. The intact epidermis rarely becomes infected. When the skin is broken by a cut, scratch, or puncture, foreign bacteria residing upon the skin, or present upon the object causing the break, are projected into the living, moist tissues, and a local or general infection may result. The healthy mucous tissues of the eye, nose and throat, and intestine resist invasion. The normal secretions upon these surfaces have mild antiseptic action. The acid gastric juice is quite a powerful disinfectant for some bacteria. The principle called bacteriophage (see page 16) is commonly present in the intestine; it is an ally of the body, assisting to defend it externally from certain infections. During the course of disease these parasites of bacteria gain in virulence and assist in recovery, and may play a part in subsequent immunity with particular diseases. Healthy, intact epithelial tissues and their secretions serve as a first line of defense.

The internal physiological defenses of the body. Once microbes have penetrated into the body proper and have lodged in the living tissues or body fluids they are immediately met with a hostile environment. Not all bacteria that gain access to the body can survive and grow there; only the pathogens can do this. The normal body fluids have a strong disinfecting (bactericidal) action toward most bacteria. If bacteria are exposed to fresh blood and then planted on a favorable medium from time to time, increasing numbers are found to be destroyed. The fresh plasma, or serum, devoid of cells, exercises this bactericidal power. Its action decreases upon

standing, and it is destroyed (inactivated) by moderate heat (55° C.) in ten minutes or longer.

Certain cells of the normal body, notably the white blood corpuscles, have the property of engulfing and destroying invading bacteria. Their action may be demonstrated by the microscopic method. This function of devouring foreign matter is called phagocytosis, and the cells are called phagocytes. Cells having amœboid motion commonly exhibit phagocytosis. It may be considered as a primitive function of certain protozoa which has been retained during all the vicissitudes of organic evolution, and which manifests itself in the amœboid cells of higher organisms as a potential protective function. It is not selective; in fact, the white blood corpuscles will engulf almost any foreign particles, organic or inorganic, which gain access to the body. They are active in the removal of degenerative material following injury or disease.

Active immunity. So far we have discussed only the natural defenses that the body possesses toward infection. These are general, or nonspecific, in nature. Of more interest is the heightened, specific resistance acquired actively by the body during disease or following vaccination. There is no demonstrable structural change accompanying immunity — we must seek the explanation in newly acquired physiological functions. Some tissues have been aroused, by the stimulation due to the foreign substances derived from the parasites, to manufacture antagonistic or neutralizing substances.

The earliest theories to explain immunity conceived the body to be composed of a stable culture medium in which certain essential food materials might be exhausted, or the waste materials of bacterial metabolism might accumulate and be retained to inhibit growth of the parasites. These were known, respectively, as the "exhaustion theory" (Pasteur) and the "retention theory" (Chauveau).

These simple explanations soon proved inadequate to explain the complex phenomenon of immunity. It became evident that the body itself is active in building up its own

defensive mechanism during infection, and retains this power for varying periods of time with different diseases, usually for the remainder of life.

Metchnikoff, studying the cellular activities of a tiny water crustacean (*Daphnia*), observed that the phagocytes were extremely active in devouring invading yeast cells in surviving animals, while they were sluggish and incomplete in those which succumbed. Upon these and other observations he built up an elaborate cellular theory of immunity. In its simplest form the phagocytic theory assumed that the experience acquired by the cells during an infection was in some way retained, so that when called upon again they reacted as a trained army rather than as raw recruits, attacking the invaders with energy and overcoming them.

There is unquestionably a parallelism between the degree of phagocytosis and the resistance of the host in many diseases. In anthrax, one of the first diseases studied, the destruction of the bacteria seems to be chiefly by phagocytes. In acquired immunity following many diseases the phagocytic power is enhanced. This does not necessarily mean that the cells have acquired any new power, however; for it might well be that the parasites are in some way acted upon by the body fluids so that their resistance toward the phagocytes is lowered. This explanation corresponds with the facts in acquired immunity, as we shall see presently.

Metchnikoff's phagocytic theory was the first attempt at a scientific explanation of immunity. It soon became evident, however, that it was inadequate to explain all the phenomena. The germicidal action of cell-free blood plasma, for example, was shown to bear some relation to the degree of immunity which an animal possessed.

The next important advance came with the discovery that certain bacteria manufactured and secreted soluble toxins which injured the body independently of the presence of any infecting agent, and that the body might establish an immunity to this poison. The immunity was specific and depended upon chemical substances, antibodies, which circulated freely

in the blood. It was even possible to transfer immunity from one animal to another by transferring a sufficient amount of the immune blood into a susceptible animal.

There could be no question about phagocytosis here, and a chemical, or "humoral," theory was advanced to explain this type of immunity.

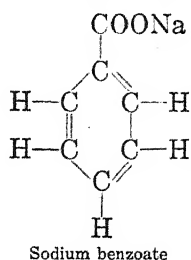
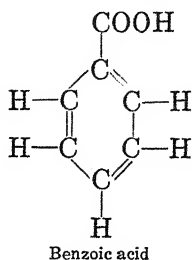
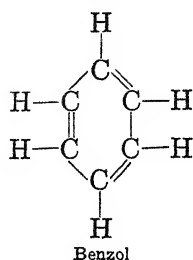
This field was rapidly extended. Only relatively few bacteria produce exotoxin in abundance. Nearly all of them extensively invade the body fluids and tissues and must be destroyed outright. Pfeiffer showed that immunity to cholera was not accompanied by the production of antitoxin, but that the blood possessed "specific bactericidal substances." These are called lysins or bacteriolysins, and exert a dissolving action toward bacteria, either within or outside of the body.

Other antibodies, agglutinins, may clump, or agglutinate, specific bacteria; precipitins cause the formation of precipitates in clear filtrates derived from cultures of organisms; opsonins act upon bacteria to prepare them for phagocytosis. The latter bodies assist in harmonizing the cellular and humoral theories, for it is evident that the two function together. White blood cells from immune animals, freed from the plasma by repeated washing, will not show any increased phagocytosis, while cells from a normal animal, treated similarly, will energetically devour bacteria that have been exposed to their specific immune serum, containing opsonin.

Ehrlich's side-chain theory. To explain the chemical mechanism involved in immunity, Ehrlich elaborated an ingenious scheme which, though inadequate to explain satisfactorily all the facts of immunity as we know them today, gives us a convenient description of the main facts.

He assumed, in the first place, that the living cells of the body must have some way of attaching and incorporating the chemical substances in the body fluids which normally nourish them. These are called *side chains*, or *receptors*, and are analogous to the bonds, or valencies, of chemical molecules which may potentially enter into union with other chemicals. Thus, in the familiar benzol ring there are the rather stable

central nucleus and six side chains, each normally attaching a hydrogen atom. One or more of these hydrogen atoms may be replaced by an acid, a hydroxyl, or other radical to form new compounds. By means of these new side chains the compound may enter into relation with still other substances, to form a number of derivatives of benzol (see Figs. 53 and 54).



If we now conceive the carbon ring as the cell body, and the side chains as more numerous and infinitely more varied, we get a notion of Ehrlich's concept. Because of their receiving function the side chains are called receptors, and graphically this relation to chemical bodies with which they may unite may be thought of as that of a lock and key.

The toxins and endotoxins of bacteria were conceived by Ehrlich to exert their injurious action by virtue of their ability to unite with these receptors. The normal metabolic functions of the cells are deranged thereby, and unless the poison can be neutralized or destroyed the cells die. But — the theory maintains — the cells do have the ability to neutralize or destroy the poisons. The attachment of these foreign chemical substances, antigens, serves as a stimulus to the generation of new receptors of the same kind, and, following a general biological law, they are generated in excess of the immediate needs. They become detached from the cell and appear in the blood as antibodies. Furthermore, the property of generating these antibodies is retained by the cells; so that, upon a slight stimulation by the same antigen a second time, the sessile receptors are rapidly thrown off into the blood stream, and promptly neutralize or overpower the invading bacteria.

Antibodies are always specific. They are as infinite in variety as the substances that may act as antigens. They are of different kinds, or *orders*, according to the nature of the demonstrable reaction which they produce when found in the blood.

The present explanation of immunity. Today there are two general types of antibodies recognized: those that react with the soluble poisons, or toxins, and those that engage with all other antigens, whether they be microbes, cells, or colloidal organic particles.

The chemical nature of antibodies appears to be closely bound up with the globulin fraction of the blood serum. The reaction between a microbe or its products and this immune principle of the blood is not a simple chemical reaction, but rather one involving surface reactions, both of the reagents being colloidal in nature. The globulin is adsorbed by the antigen, thus altering its physical as well as its chemical properties.

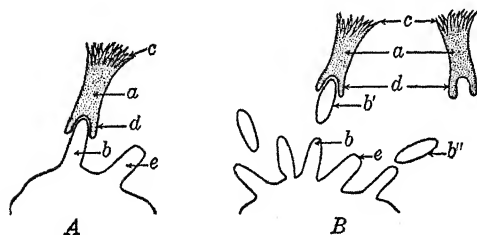


FIG. 53. Conventional diagram to illustrate Ehrlich's side-chain theory. Toxin and antitoxin

A, portion of cell with attached receptors (b, e). Receptor (b) has combined with toxin molecule (a), which is made up of haptophore (d) and toxophore (c). B, cell after stimulation, showing excess of receptors (b). Some have been liberated (b', b'') and may combine with free toxin (a) before it reaches the cell

The occurrence of a precipitate, or the clumping of bacteria, for example, is a common result of mixing antigens and specific immune serum. In such cases the surface relations are changed in such a way that the particles are impelled to draw together to form visible, or microscopic, masses. The presence of electrolytes or salts in solution is necessary in such reactions. It appears, therefore, that at first the antigen becomes coated over with the specific globulin which is present in the immune serum. This alters the electric charges that the antigens carry, and they are then flocculated by the electrolytes in which they are suspended.

In other instances the surface of the antigen, such as red blood cells, may be altered or "sensitized" by the antibody to take up a substance called *complement* which is present in all fresh blood serum, and thereby be rendered permeable so that the cell substance will diffuse out into the surrounding medium.

Local tissues appear to play an important part in removing bacteria or other foreign matter injected into the body. This is especially true if animals have previously been rendered immune. Having been arrested locally, they are taken up by the cells and digested or altered so that they are harmless.

Kinds of antibodies. There are an almost infinite variety of potential antigenic substances each of which will call forth a specific antibody response. According to the type of reaction caused, we may classify several kinds of antibodies. The antitoxins, which are in a class by themselves, neutralize the soluble poisons or exotoxins of bacteria. Snake poisons and certain other organic secretions or extracts of animals and plants behave in the same way as do such poisons or those derived from diphtheria cultures. The antitoxins are sensitive to heat and other destructive agents. They combine with toxins quantitatively, and after absorption may be caused to dissociate.

The agglutinins and precipitins cause bacteria or colloidal suspensions, respectively, to agglomerate, or floc. We have already given the explanation of this. The detection of specific agglutinins and precipitins in blood is used for the diagnosis of certain diseases. Contrariwise, the clumping of unknown bacteria by known serum may identify a culture or distinguish between strains of closely related bacteria.

The most complex reaction occurs in the lysis, or dissolving, of bacteria by immune serum. The antibody involved does not act independently, but only in the presence of a second substance in the blood known as *complement* or *alexin*. Complement is not an antibody, but is found in abundance in either fresh, normal, or immune blood. It is an unstable substance, deteriorating with age and destroyed at 55° C. The antibody is a double receptor, or amboceptor, and has a combining group for the bacteria on the one hand and one

for complement on the other. Complement is capable of acting only when the antibody, commonly called amboceptor, has been adsorbed by the bacteria or other antigenic substance. It is apparent that the complement-combining group is similar or identical, while the cell-combining group is highly specific.

The immune substances that act upon bacteria or other corpuscular elements to render them more susceptible to phagocytosis are called opsonins. This antibody globulin may

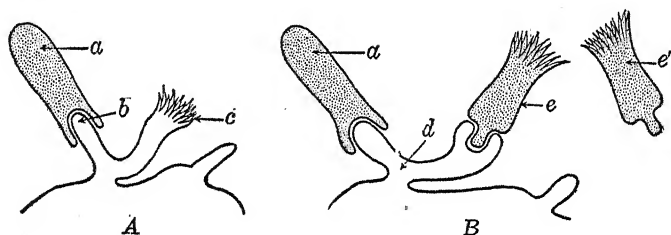


FIG. 54. Illustration of complex receptors

A represents the nature of agglutinins or precipitins. They consist of two parts: the haptophore (b), combined with antigen (a), and the zymophore (c). B represents the structure of lysins. The amboceptor (d) combines with the antigen (a), and then with complement (e). Free complement (e') is always present in excess in the blood

be pictured as coating over the cell surface to make possible the approach, engulfing, and dissolution by the phagocytic cells. Complement also engages in this reaction.

All animal cells inoculated into the body call forth the production of specific amboceptors which serve as a link between the cells and the complement. Of special interest and practical importance are the hæmolytic amboceptors, or hæmolysins, which will dissolve red corpuscles; and their production and action may serve to illustrate the general phenomenon.

If fresh, normal rabbit serum is mixed with red corpuscles of sheep, washed in physiologic salt solution, they show very feeble hæmolytic action or none at all. If the rabbit is now injected at intervals with washed sheep cells, and the blood subsequently tested in the same way, it will be found that the serum will rapidly dissolve out the hæmoglobin: the cloudy red suspension is changed to a clear red solution. If we now

substitute the red blood cells from the horse, a human being, or any other animal, no hæmolysis occurs: the amboceptors are specific and hæmolyze only the cells for which the animal is immunized, that is, sheep cells. If we heat this rabbit serum, which we may speak of as immune for sheep red cells, and add the washed sheep cells, no hæmolysis occurs. Complement has been destroyed. By centrifugation the cells may be

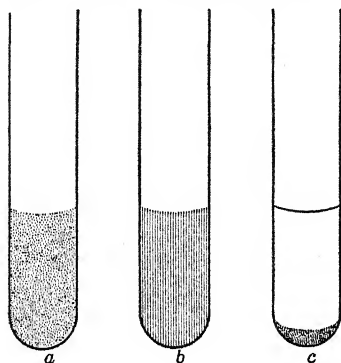


FIG. 55. Test tubes suggesting the appearances in the complement-fixation test

a, cloudy suspension of red blood cells freshly added; *b*, red blood cells laked, the fluid clear red, as in a negative test; *c*, the red blood cells at the bottom, the supernatant fluid clear, as in a positive test

thrown out of suspension, the supernatant serum removed, and the cells again suspended in salt solution. The addition of complement from any fresh serum now promptly causes hæmolysis. The amboceptors combined with the cells at the first exposure, but gave no demonstrable evidence of this until the complement was present. Such cells are spoken of as *sensitized cells*. They are used as indicators in biochemical tests, to give a color reaction to demonstrate the presence of free complement in a mixture.

Allergy and anaphylaxis. In some individuals we get marked

disturbances due to contact with certain specific substances by inhalation or ingestion. Hay-fever patients are affected by certain pollens. Some persons suffer greatly when they eat some particular food. Individuals vary greatly as to their reactions toward the inoculation of foreign serums or other organic substances. This state of natural sensitivity above the average exhibited by others is called *allergy*. It is highly specific in nature. The condition may often be alleviated by suitably graded inoculations of the substance to which the patient is sensitive, as in the case of pollen extracts used for hay fever. It is evidently an antigen-antibody reaction and

has much in common with immunity phenomena. *Anaphylaxis* (Gr., "against protection") is an induced state of hypersusceptibility. Experimentally it may be induced by injecting an animal (usually the guinea pig) with a foreign protein, such as horse serum, and then waiting for an interval of from ten to fourteen days. Upon administering a second dose of the same protein at this time a violent reaction or shock results. This anaphylactic shock appears suddenly and may result in rapid death of the animal. Death is apparently due to suffocation by the constriction of the smooth muscles surrounding the tiny blood vessels in the lungs. The reaction is specific and extremely delicate. The first dose may be unbelievably minute — as small as one millionth of a cubic centimeter in some instances.

The hypersensitive state may be transferred passively. If the serum from an anaphylactic animal is injected into a normal animal, the latter, after a suitable incubation period of from twelve to twenty-four hours, will show the typical symptoms of anaphylaxis when given an injection of the specific antigen. The substance responsible for the reaction, therefore, must be carried in the blood. As a matter of fact, the power of a serum to confer passive anaphylaxis depends upon its specific antibody (precipitin) content. It is the tissues of the animal that seem to be altered, however; for if certain smooth muscles are excised from the sensitized animal and suspended in Locke's solution, they will contract violently when even small traces of the specific antigen are added to the solution. This might indicate that the permeability, or some other property of these cells, was altered by the antibody substance in the foreign serum.

Animals once sensitized may be temporarily desensitized. If less than a fatal dose of antigen is injected into the anaphylactic animal, it will be found to be refractory, or will not react to subsequent injections, even though they be relatively massive. Persons who are naturally hypersensitive to horse serum, or who have been rendered so by previous injections given to treat or prevent some disease, may be desensitized by administering to them minute doses at the start, to be increased gradually. Any person who suffers from asthma, or

who has a history of previous serum treatment should be tested for hypersensitivity before being given antitoxin for such diseases as tetanus or pneumonia.

The treatment of animals with antigens of any sort commonly leads to increased tolerance. Under certain conditions we get exactly the opposite state, an intolerance. The mechanism involved in establishing either immunity or anaphylaxis must be similar in kind but different in intensity. If the animal is inoculated twice with the same protein at shorter intervals than ten days, it becomes refractory, or immune. A suitable incubation, or time for reaction between antigen and body, finds an unbalanced condition in which the protein, normally harmless, becomes fatally poisonous. If the animal survives the shock dose, it is thereafter refractory for a time. The animal or person once sensitized remains so to some extent for life.

The preparation of the anaphylactic animal depends upon a single stimulation of the body by an antigen acting for a suitable interval within the body. Infections ordinarily cause a continuous, increasing stimulus due to the growth of the living agents within the body and to the full release of the protective bodies. Anaphylaxis finds the same immunity mechanism developed to a point where either the cells are highly receptive to the antigen, and thereby are quickly and acutely damaged, or else the body fluids are in a condition to release a poison rapidly from the otherwise harmless substance. It would be unprofitable to pursue the theoretical considerations further in this text.

There are many practical applications of anaphylaxis. It has thrown much light on the problem of immunity itself. It helps to explain certain interesting reactions. For example, upon being vaccinated a second time we may get a prompt "take" instead of the primary slow incubation of four days preceding the first development of the papule. This accelerated reaction indicates an altered power to react, due to the presence of antibodies, and is anaphylactic in nature. The skin tests with tuberculin and mallein, for tuberculosis and glan-

ders respectively, have a similar explanation. The sensitiveness of most persons to horse serum, where injections are followed by a rash, and the occasional more violent and immediate reactions following serum treatment belong to the same order of reactions.

CHAPTER XIX

THE RESPIRATORY DISEASES

In the chapter on The Mechanism of Infection we have noted the fundamental importance of the path of entrance of a given microbe into the body in determining whether or not infection will result. The mouth and nose are the great portals into the body. Microbes are invariably present in the air that we breathe and in the food or drink that we ingest. The vast majority are harmless; but occasionally, when these products are taken into the body, they contain microbes that have recently come from infectious sources. These pathogenic organisms may lodge on the moist mucosa of the upper respiratory tract or they may be swallowed. Those that establish themselves in the nose and throat, setting up local inflammations or invading, through these tissues, the lungs or other organs of the body, we may consider as respiratory infections. The more characteristic symptoms of the disease may manifest themselves in parts of the body remote from the site of invasion. Thus, we usually think of measles, chicken pox, and scarlet fever as eruptive, or rash, diseases, because the later symptoms are in the skin. Each of these diseases gives its warning, or premonitory, symptoms in the upper respiratory tract, where the germs enter. Before the rash appears, the patient has coryza, sore throat, or inflamed eyes. In measles these first symptoms appear four or five days before the rash, when characteristic eruptions, called Koplik spots, appear in the mouth, which give a sure diagnosis. In epidemic cerebrospinal meningitis the acute inflammation occurs on the membranes of the spinal cord. The cocci that cause this disease enter and leave the body through the membranes of the posterior chamber of the nose. These diseases are all communicable during the early stages, and are most dangerous, because often unrecognized, at this time.

From the viewpoint of microbiology the respiratory diseases have nothing in common. There is no one group of organisms causing these infections; they have no relationship except this property of infecting through a particular path. From the standpoint of control or prevention they have much in common in so far as infection is concerned. They are all spread by a common medium — that is, the fresh oral and nasal secretions — and they all respond in some degree to measures which aim at the prevention of traffic in these fresh discharges. Quarantine and isolation of the sick or, in some cases, of non-immune contacts; the routine disinfection of all infectious wastes from the patient; the prohibition of spitting in public places; the abolishing of the common drinking and eating utensil; the dissemination of information regarding the importance of covering the mouth with a handkerchief when one coughs or sneezes; keeping the fingers out of the mouth; washing the hands before eating, — all these measures have some prophylactic value. No respiratory disease is wholly controlled by these measures, except perhaps in institutions, or in communities where most stringent measures are inaugurated at the very first appearance of the disease.

Complete control of this group of acute diseases is possible only by means of preventive medicine — measures, ordinarily applied by the physician, which render the individuals treated immune to specific infections. Unfortunately, prophylactic therapy is applicable today in only a limited number of diseases, but with these we have potential means of control that could be made well-nigh perfect by the intelligent co-operation of health officer, layman, and physician.

Examples of respiratory diseases. The respiratory diseases make a long list and include the common and the highly communicable group. Most of them are manifested by a sudden onset, with inflammation in some portion of the respiratory tract. They run a characteristic course and are self-limited; that is, they terminate in a fairly uniform time in convalescence and complete recovery; or else the patient dies. Recovery usually, but not always, results in immunity.

Tuberculosis is an exception to the general rules cited above. Its onset is slow and insidious, and the symptoms are vague. It is chronic and not self-limited; and while antibodies are developed and light infections apparently heighten resistance, there is never immunity in the usual sense of the term.

Tuberculosis is one of the most prevalent and fatal infectious diseases of mankind. It has been appropriately called the "great white plague." It is responsible for more than seventy-five thousand deaths annually in the Registration Area of the United States. Ninety per cent of the cases are pulmonary infections. The organism may, however, attack almost any organ of the body: joints, nervous system, glands, or the internal organs generally. The bovine type of the tubercle bacillus, spread from cows through milk, may be infective for children, especially under the age of five years.

Robert Koch described the organism and proved its causative relation in 1882. The tubercle bacillus (*Mycobacterium tuberculosis*) (see Frontispiece), is a delicate, slender, slightly curved rod, frequently occurring in characteristic clumps. Occasionally it shows branching; hence its classification with the higher bacteria. The cell wall contains a wax, or fatty substance, which makes it difficult to stain; but once it becomes stained it will not decolorize when treated with weak mineral acids in alcohol. It is therefore called an *acid-fast* organism. Laboratory diagnosis depends upon this property.

The organism appears in vast numbers in the sputum of active pulmonary cases, and is spread chiefly by this material. It is more resistant than most pathogenic bacteria to drying, heat, and other disinfecting agents.

Tuberculosis is curable if treatment is begun in time. It is of supreme importance that it be diagnosed early. This can be assured only by the use of the tuberculin test and the X-ray. Tuberculin is a specially prepared extract from tubercle bacilli which causes a characteristic local reaction when introduced into the skin of persons who have tuberculosis even in the very early stages. When a positive tuberculin test is found, the X-ray picture will usually reveal the location and

extent of the infection. Infection plays a major rôle in infancy and early childhood, but the disease is held in abeyance in later life by the practice of good personal hygiene.

Pneumonia is second only to tuberculosis as a fatal infectious disease. While tuberculosis is a chronic disease, showing a fairly uniform rate year in and year out, pneumonia shows

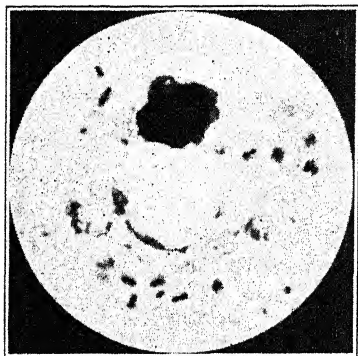


FIG. 56. *Diplococcus pneumoniae*

Photograph by Zettnow

marked fluctuations. The incidence of other epidemic diseases, notably influenza, has a marked effect on the incidence and fatality of pneumonia.

Secondary pneumonia, usually broncho-pneumonia, is the dreaded ally of other diseases. The resistance having been lowered by measles, grippe, or other causes, the lung tissues become susceptible to invasion by bacteria, most commonly streptococci, that may cause inflammation in these tissues.

Pneumonia is not a distinct disease entity, but includes a variety of affections of the lungs. The most common micro-organism found in primary lobar pneumonia is a slightly elongated, Gram-positive coccus, usually surrounded by a rather thick capsule, and occurring in pairs, or sometimes in chains (see Frontispiece). This is the *Diplococcus pneumoniae* or pneumococcus. Streptococci, staphylococci, the Friedländer bacillus, and other bacteria may also cause pneumonia.

The organisms belonging with the typical pneumococci by morphological and cultural tests are not all identical in their pathogenic properties. When tested by certain immunity reactions, as by agglutination or precipitation, or the swelling of the capsule in the presence of the blood serum of specific immune animals, the pneumococci can be divided into four rather distinct groups or types. These are designated as Types I, II, and III, and Group IV. The most severe pneu-

monias are produced by the first three types. Group IV is a composite group, including at least twenty-nine types (Types IV-XXXII). These organisms are frequently found in healthy mouths and are of relatively low pathogenic power. Types I and II are rarely found in healthy mouths except among those who have been in contact with cases of pneumonia.

TABLE XXIV. PERCENTAGE OF POSITIVE TYPE I AND TYPE II PNEUMOCOCCUS CULTURES IN THROATS OF CONTACTS AND NONCONTACTS¹

PNEUMOCOCCUS	CONTACTS		NONCONTACTS	
	Number Examined	Per Cent Positive	Number Examined	Per Cent Positive
Type I	160	13	297	0.3
Type II	149	12	297	0.0

One does not acquire any permanent immunity upon recovering from pneumonia. Antibodies must be produced to explain recovery, and we have noted that there are specific substances in immune blood that react with the organisms or their products. There is an antiserum for Type I which is of great value in treating Type I infections. The antisera for the other types are feeble and uncertain in treating human infections, but there is considerable promise that with Type II, at least, a reliable antiserum will be developed.

Measles, mumps, chicken pox, and whooping cough are sometimes spoken of as the "common communicable diseases of childhood." They are chiefly diseases of early life, but adults are apparently immune only because the majority have had these diseases earlier.

Measles is one of the most highly communicable diseases of mankind. It is spread by direct contact exclusively. The most contagious period precedes the appearance of the rash by several days. There are no carriers, and the convalescing patient will not spread the disease.

The disease is far more fatal than is ordinarily appreciated. In 1923 it caused three times as many deaths as scarlet fever

¹ E. O. Jordan, General Bacteriology.

and nearly as many as diphtheria. Deaths occur chiefly under the age of five, and are concentrated in the first three years of life. Fatal issue is due to pneumonia superimposed on the system weakened by measles.

The cause of measles has usually been described as a filtrable virus of unknown nature. Recently a "green-producing" diplococcus has been associated with the disease. This organism is uniformly found in the blood during certain stages of the disease and has been isolated from the nose and throat. The organism has been cultured and will reproduce the symptoms of the disease. Extracts from cultures cause local irritation in persons susceptible to measles and not in immunes. Serum from recovered patients has germicidal power for this coccus. These results have not been generally confirmed, and the virus theory is still generally held.

Whooping cough is likewise a serious disease in early life. Babies and young children should receive every possible protection from infection; but if infection does occur, they should have immediate medical and nursing care.

The Bordet-Gengou bacillus (*Hæmophilus pertussis*) is associated with this disease. It infects the ciliated surface of the trachea, the irritation causing the characteristic paroxysmal cough. This organism is agglutinated by the serum of the recovered patient. Pertussis vaccines prepared from freshly isolated cultures have been developed which have preventive and curative value for whooping cough.

The causative organisms of *mumps* and *chicken pox* have not been proved. They are both spread by fresh mouth discharges, and chicken pox may also be spread by the scab or scale from the healing skin lesion.

Scarlet fever is a dread disease, and rightly so. It has shown a tendency of late to become increasingly mild, and as a cause of death it does not rank among the first. Even light cases may, however, leave the patient with impaired hearing or vision, damaged kidneys, or other serious sequelæ.

The organism causing scarlet fever has been exceedingly obscure and difficult to prove. Recent work, dating from 1923,

seems finally to have confirmed the suspicions of earlier investigators, for it is quite firmly established that a hæmolytic streptococcus is the cause. Streptococci have repeatedly been found associated with scarlet fever infection, but the Doctors Dick of Chicago first cultivated the organism in pure culture and succeeded in reproducing symptoms resembling the disease in human volunteers. A toxin has been recovered from bacteria-free filtrates which will cause local inflammation in persons who have never had scarlet fever, but not in immune persons (the Dick test). This toxin is neutralized by the serum of scarlet fever convalescents. This test now gives a method of determining susceptibility to the disease.

Dochez, working with a hæmolytic streptococcus, arrived at the same conclusion as to the cause of scarlet fever by a different method. It has been observed that the human serum from a convalesced patient inoculated into the skin in the region of the scarlet rash in an active case of the disease would promptly blanch, or bleach, the rash. Dochez inoculated horses with a streptococcus culture recovered from a case of the disease, and after a suitable period he found that the serum from the horse thus immunized would cause this characteristic whitening of the skin in scarlet fever patients. It was evident that there must be similar or identical antibodies in human serum and horse serum, and that therefore the same antigen, the streptococcus, must have stimulated their production.

An antitoxin for scarlet fever is now manufactured which is a reliable curative agent. Furthermore, it is possible to actively immunize children or others with small amounts of the toxin recovered from streptococcus cultures and with a detoxified toxin known as toxoid.

A few examples of infections entering and leaving the body through the respiratory tract have been selected for consideration. This list is far from complete, but we may most profitably devote the remainder of the chapter to a more detailed discussion of one of the most important diseases of the group—the disease about which our knowledge is most complete, and where, as a result, the possibility of control is greatest.

Diphtheria and its prevention. *Diphtheria* is an acute, highly contagious disease, characterized by a sudden onset, with sore throat, fever, and other general symptoms. It is a disease chiefly of early childhood, the great majority of cases occurring between the ages of six months and five years. It is a highly fatal disease: in severe epidemics as many as twenty-five per cent of untreated cases result fatally. On the other hand, diphtheria may run a very mild course and may even go unrecognized, passing for an ordinary sore throat or tonsillitis. Such "missed cases" are very dangerous to the public health.

By the intelligent and general use of the knowledge that we possess of diphtheria not only could all deaths from this cause be prevented, but within ten years the disease itself could be made practically obsolete. That this goal is attainable is indicated by the results in Massachusetts, where in 1923 there were 9000 reported cases and 579 deaths from diphtheria as against 390 cases and 26 deaths in 1935. Understanding, coöperation, and facilities for immunization will render this disease "as obsolete as the dodo." Let us summarize what we know of diphtheria and its prevention: (1) We know the organism causing the disease, and can recognize it by direct microscopic examination. Therefore (2) we can confirm clinical diagnosis; (3) we can establish rational quarantine of contacts; and (4) we can isolate convalescent patients until after the organisms have disappeared from the nose and throat. (5) Patients can be almost certainly cured by the prompt use of antitoxin. (6) Contacts, and persons in institutions, may be temporarily protected in emergencies by passive immunizing with antitoxin. (7) We can differentiate the non-immunes from the immunes by a simple, safe skin reaction known as the Schick test. (8) Finally, and most important, we can safely immunize all susceptible persons by the injection of neutralized mixtures of toxin-antitoxin or of toxoid. To summarize, we can detect persons who are most apt to spread the disease; we can cure the developed cases; and we can immunize temporarily, in emergencies, or permanently immunize, all who are susceptible to diphtheria.

The diphtheria organism was first described by Klebs in 1883, and its etiologic, or causal, relations were proved by Löffler in 1884. It is sometimes called the Klebs-Löffler (K-L) bacillus, after its discoverers.

The diphtheria bacillus (*Corynebacterium diphtheriæ*) is a slender rod from $1\ \mu$ to $6\ \mu$ in length by $0.3\ \mu$ to $0.8\ \mu$ in diameter. It is usually slightly curved, and is swollen at one or both ends or in the middle. It stains irregularly, usually showing deeply staining metachromatic granules in the swollen portions, but sometimes being barred.

The cells are often clumped, and it is characteristic to find two cells forming an obtuse angle with one another, V-shaped, or three cells in a Y shape. Branching forms are sometimes observed. There is a marked tendency to produce involution forms (for illustrations see Fig. 14 and Frontispiece).

The bacillus grows readily on suitable culture media, especially upon blood serum containing 1 per cent of glucose and in a veal broth. It is aërobic and facultative. Toxin is produced best under good aërobic conditions in a slightly alkaline medium.

The diphtheria organism secretes a soluble *exotoxin*, and the disease manifestations are due chiefly to this toxin. This poison can be freed from the bacilli by filtration and acts entirely independently of them. It is destroyed by heat and by sunlight, and degenerates slowly up to a certain point upon aging; but thereafter, if kept cold, it is fairly stable. Inoculated into animals in sufficient amounts, it causes the same symptoms that an infection would produce, and it stimulates the immunity mechanism to antitoxin formation. Upon this fact rests the manufacture of antitoxin by inoculating horses or goats, and also the production of active immunity in man.

The only *natural habitats* of the bacillus are the mucous surfaces in man — chiefly the nose and throat. Natural infections have been described in cats, but they are certainly very rare and of no practical importance. This disease is spread chiefly by direct contact of persons harboring virulent organisms with healthy susceptible persons. It may be spread also

by milk. Fomites, domestic animals, and insects do not disseminate diphtheria, as is sometimes claimed. Morphologically and culturally typical organisms show wide fluctuations in pathogenicity and in their ability to produce toxin. There seem to be avirulent and virulent strains. The nonvirulence of typical bacilli found in healthy noses and throats adds a complication to the control of carriers.

Persons may harbor virulent cultures under the following conditions: in typical cases; in convalesced cases (for an indefinite period); in light, unrecognized cases; in the case of healthy immune contacts; and in the case of healthy carriers that may or may not be immune as shown by the Schick test. The difficulty of detecting and controlling all these groups is too great to be practicable. The cases and the contacts are most important, and these should be isolated until at least two successive negative cultures are found in the laboratory.

Behring and Kitasato discovered *antitoxin* for diphtheria in 1890. In 1894 its manufacture and use for the treatment of human cases became practical. This marks the modern era of the conquest of diphtheria and is a landmark in preventive medicine. Wherever its use was introduced, the mortality rate was reduced by 50 per cent or more. In New York, for the ten-year period 1885-1894, the death rate per ten thousand from diphtheria was 15.19. For the period 1895-1904 it dropped to 6.62. For the same periods in Boston the rates were 11.76 and 6.34 respectively. These declines are typical.

In 1913 Dr. Bela Schick described the *Schick test*. This is performed by inoculating a minute amount of pure diphtheria toxin between the layers of skin on the forearm. In the susceptible person with less than a certain minimum amount of free antitoxin in the circulation there will develop a characteristic local inflammation at the site of inoculation, while in the immune no reaction occurs. The following table shows the percentage of persons in different age groups, in a typical city population, who give positive Schick tests.

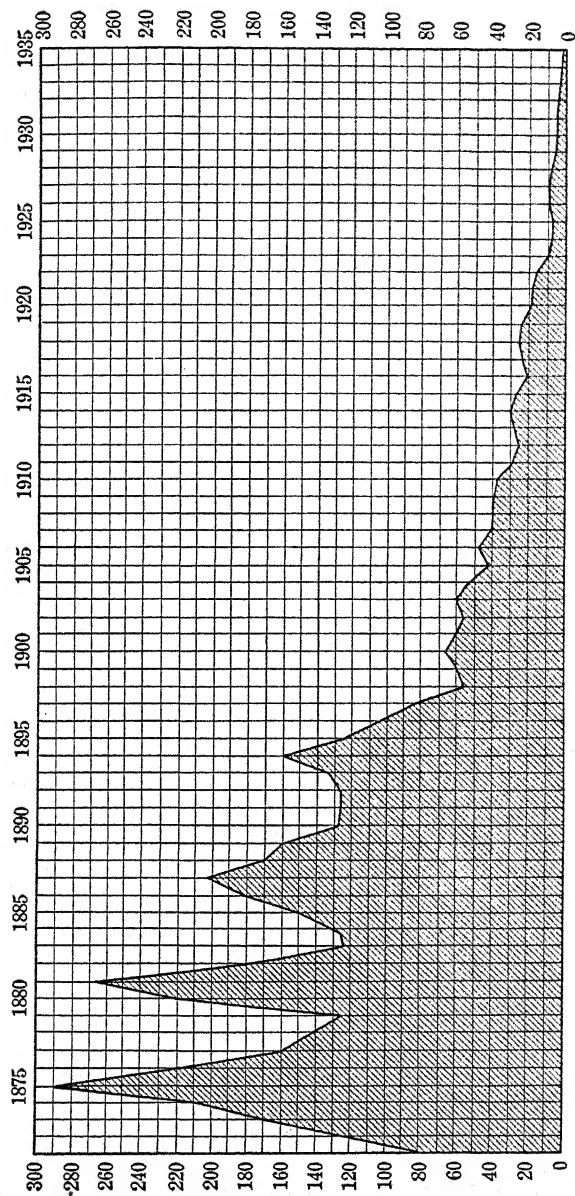


Fig. 57. Death rate from diphtheria and croup per 100,000 population in New York City from 1871 to 1935
(Extended from *Monthly Bulletin*, Vol. XIV, No. 2, New York City Department of Health)

TABLE XXV. PERCENTAGE OF PERSONS SUSCEPTIBLE TO DIPHTHERIA (AS INDICATED BY THE POSITIVE SCHICK TEST) IN NEW YORK CITY ¹

AGE	PER CENT SUSCEPTIBLE
Under 3 months	15
3-6 months	30
6 months to 1 year	60
1-2 years	60
2-3 years	60
3-5 years	40
5-10 years	35
10-20 years	25
20-40 years	18
Over 40 years	12

The Schick test would be of no great prophylactic value if we did not have a means of immunizing those persons, particularly preschool children, who prove to be susceptible. Fortunately we have a safe, reliable means of actively immunizing those who are susceptible, by the use of diphtheria toxin neutralized with antitoxin or of toxoid prepared by treating toxin with formaldehyde.

The toxin-antitoxin mixture is inoculated, in accurate amounts, in three successive doses at intervals of a week. Toxoid is to be preferred for the immunization of children under ten years of age; it is administered in three doses, preferably at intervals of three weeks. Immunity develops slowly (within one to six months usually) and lasts for eight years and probably for the remainder of life. This is the most important single weapon that we have to combat diphtheria.

Diphtheria today is as preventable as is smallpox. If all preschool children could be immunized, the disease would become rare or obsolete. It will take a generation, or many generations, to overcome all prejudices; and in the meantime some little children will be sick with diphtheria, and some will needlessly die of this disease. But with an increasing proportion immunized, the prevalence has been, and will be, still further reduced. This is the heritage that we have received from a long line of painstaking investigation.

¹ W. H. Park, in *Journal of the American Medical Association*, 1922, Vol. 79, p. 1587.

CHAPTER XX

THE ALIMENTARY DISEASES

The alimentary or intestinal diseases include those caused by microörganisms which infect the body through the mucous membranes of the intestinal tract and which escape from the body chiefly with the wastes from this origin. They are characteristically filth-borne. The first, and ordinarily the most prominent, symptoms are gastrointestinal in nature, although other organs, or the whole system, may be invaded by the parasites.

The alimentary diseases are not primarily spread by contact (contact-borne), as is the case with the respiratory group. They may be spread directly from person to person because of unclean habits, but they are chiefly vehicle-borne. Water, milk, shellfish, and other foods that are frequently eaten without a preliminary cooking, or foods that are carelessly exposed and handled a good deal, serve as the medium for the distribution of this group of disease germs. Flies or other vermin that come in contact with food may serve to infect it. Foods that are contaminated and then improperly kept, or kept too long, may become heavily seeded with microörganisms and cause very sudden acute infections, the so-called food poisonings.

Besides bacteria that invade through the intestinal tract there are also animal parasites that use this portal. The tiny trichina, occurring in uncooked or improperly cooked pork products, bores through the intestinal wall and lodges in the muscles, causing a distressing and often fatal disease called trichinosis. The tapeworm and hookworm are characteristic intestinal parasites. Hookworm is one of the most ubiquitous parasites of man, occurring in a large proportion of all rural dwellers in the warmer countries of the earth. The eggs of this tiny worm escape from the body with the alvine wastes of the

infected person. They germinate in the soil, entering the new host through the skin, usually through the feet. By a circuitous path they finally arrive in the intestine of the new host and begin the life cycle over again. Elementary environmental sanitation, and proper medical treatment of cases to eliminate the parasites from the intestine, will rapidly reduce the prevalence of this disgusting disease.

The colon-typhoid group as parasites. The colon-typhoid group of bacteria have already been discussed in relation to water bacteriology. The pathogenic bacteria causing intestinal diseases, including typhoid, the paratyphoids, and dysentery, and a group of organisms causing acute food poisonings belong to this group. The organism causing cholera (*Vibrio comma*), although similar in distribution and in its modes of infection, is not a close relative of this group.

The colon-typhoid group has been described as including non-spore-bearing, Gram-negative, short rods (sometimes occurring as long filaments), which grow fairly well on culture media, forming typical surface colonies on gelatin, and showing graded fermentative properties for carbohydrates as we pass from one extreme of the group to the other. Most of them are intimately associated with animal life, and several are typical parasites. The balance between saprophytism and parasitism with several members of the group is a delicate one; and at a favorable opportunity, as in intestinal perforation, or lowered resistance of the host, they may become parasitic.

In their habits, and sources and modes of infection, the several members are so similar that a discussion of the most important one, typhoid, in some detail will give a good general knowledge of the group.

The typhoid bacillus (*Eberthella typhi*) was discovered in 1880 by Eberth, and in 1884 Gaffky succeeded in growing this organism in pure culture. The disease is not readily transferred to laboratory animals except by injections into the blood stream or peritoneal cavity, so that the proof of its causal relationship was slow in accumulating. The constant occurrence of the organism in the intestines of patients,

and serum reactions between the patient's blood and typhoid cultures, together with other experimental evidence, are overwhelmingly convincing. Today it is accepted as the specific cause of typhoid fever.

The bacillus is from $1\ \mu$ to $3\ \mu$ long by $0.5\ \mu$ to $0.8\ \mu$ in diameter. In young, vigorous cultures long filaments are common. It is actively motile, possessing peritrichous flagella.

The cultural characters have been discussed (see Fig. 41).

Isolation of the typhoid bacillus is essential in detecting carriers and in the release of patients. The chief difficulty is to separate typhoid bacteria from the colon and related bacteria that are always present in large numbers wherever typhoid is found. The media devised usually contain selective dyes which inhibit the growth of most bacteria except typhoid and give a differential growth for this organism. The details of methods of isolation are discussed in the last chapter.

The typhoid bacillus is an exclusive parasite of man. It never infects other animals in nature, and its occurrence outside the human body is purely accidental. It multiplies only within the host, unless possibly in milk under favorable conditions of temperature. Its survival outside the body depends upon many factors, but it rarely lives more than from a few days to three or four weeks.

In the human body the typhoid bacillus is found in the dejecta, in the urine, and, in the early course of the disease, in the blood. It is occasionally found in the saliva. The bile duct is frequently infected, and often infection persists here for a long time and explains the origin of a good proportion of the chronic carriers.

The patients are sources of infection during the course of the disease and for an indefinite period following convalescence. From 2 to 4 per cent become carriers for ten weeks or longer; some, probably for life. There are apparently light or missed cases, as examples are cited of carriers who have no history of having had typhoid.

Immunity is commonly acquired after typhoid fever, although there are many authentic examples of persons who

have experienced more than one typical attack. Antibodies develop in the blood early in the course of the disease, and the demonstration of agglutinins has diagnostic value (see the Widal test, p. 274).

Vaccination against typhoid fever by the use of graded inoculations of pure cultures of bacilli killed by heat has had wide application, especially in armies, and has proved of the greatest prophylactic value. Since 1911, when typhoid vaccination was made compulsory in the army, the disease has practically disappeared.

TABLE XXVI. RATE OF TYPHOID FEVER IN THE UNITED STATES ARMY FOR THE FIVE YEARS PRECEDING AND THE FIVE YEARS FOLLOWING COMPULSORY VACCINATION

YEAR	NUMBER OF CASES	RATE PER THOUSAND	NUMBER OF DEATHS	RATE PER THOUSAND
1906	347	5.66	15	0.28
1907	208	3.53	16	0.19
1908	215	2.94	21	0.23
1909	173	3.03	16	0.28
1910	142	2.32	10	0.16
1911 ¹	44	0.85	6	0.09
1912	18	0.31	3	0.04
1913	4	0.04	0	0.00
1914	7	0.07	3	0.03
1915	8	0.08	0	0.00

It is evident, from an inspection of this table, that not only the case rate but also the fatality rate is reduced by vaccination.

The control of typhoid by sanitation. In groups exposed to special risks of infection, as armies, or in civil populations menaced by the presence of typhoid epidemics, vaccination is an important preventive measure. In civil communities generally, vaccination should be unnecessary, for sanitation should be so thorough that outbreaks of this filth disease are impossible. A continuing high typhoid rate in a city to-day is an indication of a primitive, uncivilized condition, and is a disgrace. Occasional sporadic outbreaks, due to human

¹ Compulsory vaccination against typhoid inaugurated.

fallibility, may still occur; but such epidemics can ordinarily be stamped out promptly, and safeguards against their recurrence from the same cause can be established.

The group of infectious intestinal diseases in general are amenable to control by public sanitation. Freedom from these diseases can be bought for a price. In the larger communities the individual citizen is protected without any conscious effort on his part, except that he sanctions the expenditure of a modest portion of his taxes for safeguarding his water, and his milk and other food supplies, from infection with sewage bacteria. He rarely has misgivings or "conscientious objections" concerning this, so that the health officer or sanitary engineer does not have to combat the personal equation to promote this part of the public-health program. That is not to say that there is no resistance offered to sanitary progress. The pasteurization of milk has been vigorously resisted, and all too often communities must first experience the tragedy of a water-borne outbreak of typhoid before they will act upon warnings of the menace of polluted water that have been given for years. In rural communities the householder is himself largely responsible for waste disposal and water supply and must be urged or required to protect himself and family from these diseases.

The acute food-infection group. Closely related to the true typhoid bacillus is the *Salmonella* group of bacteria, including a number of species that are parasitic in man and in lower animals. Several of these may be responsible for acute food infections which will be discussed presently. The *Salmonella schottmülleri* (formerly called paratyphoid B) and to a less extent *Sal. paratyphi* (A) cause the paratyphoid fevers. In their sources and modes of infection and the general symptoms caused they closely resemble typhoid fever. The disease may be differentiated by agglutination tests with the blood of the patient, or by culture reactions.

In their fermentation reactions the *Salmonella* group is intermediate between *Escherichia* and *Eberthella*. They do not produce gas in lactose, but ferment dextrose and mannite with gas production. The relationship may be shown in a table.

FERMENTATION IN

ORGANISM	DEXTROSE	LACTOSE	MANNITE	XYLOSE
<i>Eberth. typhi</i>	A + G -	A - G -	A + G -	A ± G -
<i>Sal. paratyphi</i>	A + G +	A - G -	A + G +	A - G -
<i>Sal. schottmülleri</i>	A + G +	A - G -	A + G +	A + G +
<i>Esch. coli</i>	A + G +	A + G +	A + G +	A + G +

A = acid; G = gas

Water-borne diseases. Water is the most important vehicle for the dissemination of typhoid and other intestinal diseases. The wastes of the body (sewage) sooner or later find their way into watercourses. Municipal sewage will ultimately find its way into some neighboring body of water in which it is diluted and carried away. This may be a safe practice when the elimination is made into sea water; but when infectious wastes enter lakes, rivers, streams, or wells, they may endanger drinking water. The microbes survive for two or three weeks, which gives time for them to be transported with currents, to reach some water intake, and to be distributed through the water mains to the drinking glass. It has been estimated that up to within the last fifteen years from 50 to 75 per cent of the deaths from typhoid in American cities were due to polluted water; in other words, communities with high typhoid-fever death rates could, by eliminating typhoid bacteria from the water, anticipate saving at least from five to seven lives out of every ten which would otherwise be lost from this cause each year. The experience with water purification amply justifies this estimate. Albany, Binghamton, Cohoes, and Niagara Falls, all in New York State, filter and chlorinate their water. The following table shows the results:

TABLE XXVII. AVERAGE TYPHOID-FEVER DEATH RATE PER HUNDRED THOUSAND OF POPULATION BEFORE AND AFTER TREATMENT OF WATER

CITY	FIVE-YEAR AVERAGE BEFORE TREATMENT	FIVE-YEAR AVERAGE AFTER TREATMENT
Albany	89	5.6
Cohoes	99	3.5
Binghamton	56	3.7
Niagara Falls	132	5.3

Water-borne diseases may be prevented in three general ways: (1) by diverting the infectious sewage into some other channel; (2) by obtaining a new supply from an unpolluted source; (3) by treating, or purifying, the infected supply. Chicago improved its water supply by diverting its own sewage from Lake Michigan into the great drainage canal. Lowell substituted a pure upland water for the polluted Merrimac River water. Lawrence elected to purify the Merrimac River water.

Water purification usually presents a solution of the problem if the supply is not too grossly polluted. The impounding, or *storage*, of a water improves its sanitary quality owing to the elimination of bacteria by natural causes. Subsidence, sunlight, and the absence of the appropriate oxygen, food, and tempera-

ture relations tend rapidly to reduce sewage bacteria, especially the pathogens. This method of purification is unreliable and should be fortified by filtration or chlorination when the water is known to be contaminated.

Filtration is performed by passing the water through specially prepared sand beds that are thoroughly underdrained. In the *slow sand filter* the water is applied to the surface of the sand and passes vertically downward through the filter. A little reflection will make it evident that the spaces between the sand grains must give ample opportunity for bacteria readily to pass through the filter unless some factor other than the straining, or filtering, action of the sand is involved (see Fig. 58). Bacteria that may be readily recovered and recog-

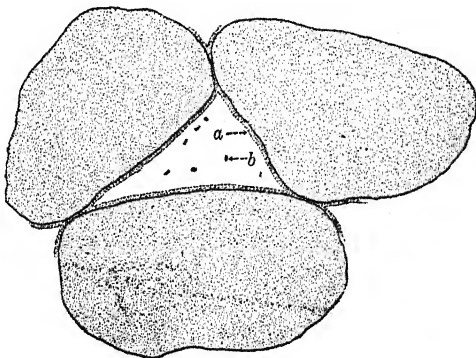


FIG. 58. Figure to show the approximate relative size of sand grains, interspaces, and bacteria in a filter

a, gelatinous growth; b, bacteria

nized, such as *Serratia marcescens*, may be added to the raw water applied at the top of a new filter just put into operation, and samples collected at intervals in order to detect their appearance in the effluent. The water begins to percolate through in a few minutes, but the bacteria added to the water do not appear for hours. The filtered water contains only a fraction of the number in the raw water. Evidently there is a purifying influence due to some factor other than that of straining, or filtering, in the ordinary sense. In the slow sand filter, after it has been used for a few hours, a gelatinous growth and deposit appears on the sand grains, especially on those near the surface, reducing the free interstices between the sand grains and giving a colloidal matrix which will tend to attract and retain minute suspended particles, including bacteria. Bacterial antagonism, or possibly bacteriophagy, may play a part in the reduction and destruction of bacteria. There is apparently some selective destructive action for the sewage forms, as bacteria of the colon group show a disproportionate reduction. This problem needs some further careful investigation.

Phelps graphically explains the theory of slow sand filtration in the following terms:¹

A cubic foot of an ideal filter sand, composed of perfect spheres of uniform diameter and of an average water-filter size, 0.3 millimeters in diameter, has a superficial area of 4500 square feet of sand surface exposed to the water. This same cubic foot has a water capacity of 0.26 cubic foot; so that the average depth of the water layer over the entire surface of the sand grains is 0.0007 inch, or roughly 17 microns. The length of a colon bacillus is about one micron. The water flows in this extremely thin layer through a tortuous channel, and the eddy currents produced are probably as violent in comparison with the depth as they are in a mountain stream flowing swiftly over a rocky bed. The chances of direct contact between a single bacterium and the surface of a sand grain are therefore great. The sand grains are coated with a gelatinous mixture of deposited organic matter and living bacteria. They have the property of colloidal materials in general to "gather," or agglutinate, micro-

¹ Earle B. Phelps, Public Health Engineering, p. 94. 1925.

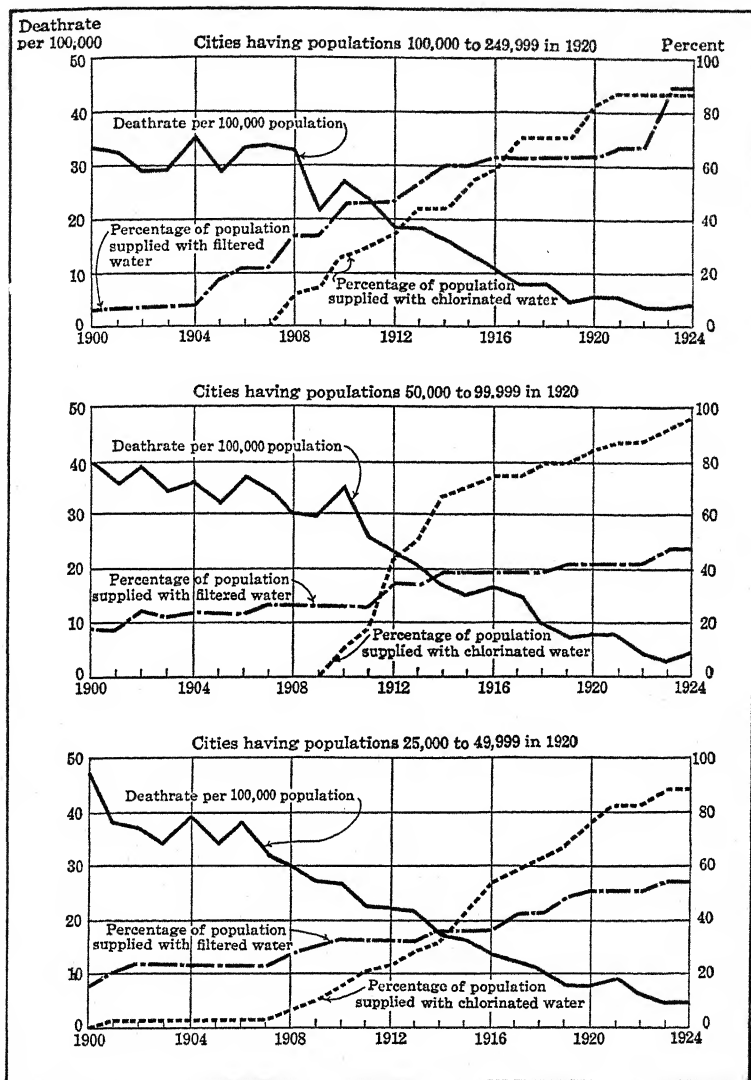


FIG. 59. Typhoid fever control through filtration and chlorination of water supplies in American cities, 1900-1924. By size of city¹

¹ Metropolitan Life Insurance Company, Statistical Bulletin, March, 1927.

scopic particles. This may be only a temporary effect in the case of an individual organism, but the result of repeated retardations of this sort is evidently sufficient to delay the passage of organisms for hours or days, according to the laws of chance. Many of them are permanently held; and their bodies go to make up the gelatinous residue with which the grains of sand are coated and upon which other bacteria, more at home in this environment, subsist.

Rapid, or mechanical, filtration depends upon a preliminary clarification and partial bacterial removal by the formation of a colloidal gelatinous precipitate formed by the addition of aluminum sulphate to the water. When the "alum" is added to water, it is decomposed to a hydroxide which forms tiny flakes that agglomerate into larger masses and sink, carrying down suspended foreign matter, including many of the bacteria present. The water is applied to the filter, and enough of the flocculent matter remains actually to clog the pores between the sand grains and to strain, or filter, out most of the remaining bacteria.

The *disinfection* of water is the latest, and probably the most important, development in water purification. The requirements of a satisfactory agent for destroying bacteria in water are not easily satisfied. Apart from being a reliable disinfectant, it must have no deleterious physiological effects when taken into the system with drinking water; it must not corrode pipes or boilers; it must be tasteless, odorless, and colorless; it must be cheap, stable, and readily applied at a uniform rate. Of all the agents thus far tested, only one, chlorine (together with the hypochlorites), has met all the requirements. Chlorination today is the most widespread method of water treatment, being used in combination or alone by over ten thousand plants in operation in America. The required dosage is extremely minute. It disinfects either by the liberation of nascent oxygen from water or by the direct toxic action of chlorine or the hypochlorous acid which is formed. The action is selective, bacteria being attacked more readily than inert organic matter that may be present; and the least-resistant bacteria in the water, ordinarily the colon-typhoid

group, are first destroyed. The most appropriate use of chlorination is as an auxiliary to other methods of water purification rather than as an independent system.

The influence of chlorination upon typhoid death rates is comparable to the favorable results shown by other methods.

TABLE XXVIII. EFFECT OF CHLORINATION ON TYPHOID DEATH RATES¹
(AVERAGE RATE PER 100,000 OF POPULATION)

CITY	COMMENCED CHLORINATION	BEFORE USING		AFTER USING		PERCENTAGE OF REDUCTION
		Period	Rate	Period	Rate	
Baltimore . . .	June, 1911	1900-1910	35.2	1912-1915	22.2	36
Cleveland . . .	Sept., 1911	1900-1910	35.5	1912-1916	8.2	77
Erie	Mar., 1911	1906-1910	50.6	1912-1914	15.0	70
Jersey City . .	Sept., 1908	1900-1907	18.7	1908-1916	8.4	55
Kansas City . .	Jan., 1911	1900-1910	42.5	1911-1916	14.2	66
Trenton	Dec., 1911	1907-1911	46.0	1912-1914	28.7	35
Toronto	Apr., 1911	1906-1910	31.2	1912-1916	7.8	75
Ottawa	Sept., 1912	1906-1910	34.0	1913-1917	17.0	50

Milk. Next in importance to water as a vehicle of typhoid and other intestinal diseases comes milk. Milk is most likely to be infected with typhoid by a carrier, either at the dairy or at some point where it may come in physical contact with milk-handlers. The outbreaks are usually explosive in nature, a large number of cases appearing in a brief space of time; and the cases show a distribution corresponding to a particular milk route.

The sanitary production and handling of milk, followed by pasteurization for all city supplies at least, will largely eliminate epidemic milk-borne diseases. While pasteurization is not infallible, it is the nearest to a foolproof method that we yet have; and, duly performed, under official control, it does prevent disease. The table on page 268 shows the decline in milk-borne diseases in Massachusetts. In 1919 approximately 34 per cent of the milk was pasteurized; in 1931 it had increased to 79.5 per cent. Septic sore throat, first made reportable in 1914, is seldom recognized except in epidemic forms, and most epidemics are milk-borne. Milk-borne diphtheria

¹ Race, Chlorination of Water.

has disappeared, and scarlet fever is approaching the vanishing point. The percentage of cases of typhoid traced to milk during the period 1930-1932 was insignificant; but as long as there is any raw milk consumed, there remains the danger of milk infection by healthy carriers.

TABLE XXIX.¹ PERCENTAGE OF TOTAL CASES OF CERTAIN DISEASES TRACED TO MILK

PERIOD	SEPTIC SORE THROAT	TYPHOID FEVER	SCARLET FEVER	DIPHTHERIA
1896-1900 . .	—	2.1	0.0	0.1
1901-1905 . .	—	1.9	0.6	0.0
1906-1910 . .	—	8.1	3.8	0.2
1911-1915 . .	—	5.3	1.2	0.1
1916-1920 . .	75.5	6.9	0.4	0.1
1921-1925 . .	10.1	6.7	0.2	0.1
1926-1929 . .	58.3	8.3	0.3	0.0
1930-1932 . .	27.6	0.4	0.002	0.0

In addition to epidemic diseases, milk improperly produced and manipulated is a chief cause of diarrheal diseases in infants. The infant-welfare program in America started with a project to furnish babies with clean milk, free from high numbers of bacteria. Fortunately pasteurized milk is as good for the average baby as is raw milk, and is to be preferred from the standpoint of safety alone. The decline in infant mortality is one of the most striking phenomena of the age (see Table XXX); and though many other factors have played a rôle,—notably the instruction of mothers by public-health nurses,—the elimination of dirty, germ-laden milk is of first importance.

Shellfish. Shellfish, principally oysters, are grown in tidal waters that oftentimes in the past have received raw sewage. They may be used as food without a preliminary cooking, and therefore may serve as a vehicle for living sewage bacteria.

During the latter part of the year 1924 there was a striking increase in the incidence of typhoid fever in a number of widely separated cities in the United States. The outbreak

¹ Bigelow and Feemster, in *American Journal of Public Health*, Vol. XXIII (1932), No. 6.

TABLE XXX. INFANT MORTALITY IN MASSACHUSETTS, 1905-1934

YEAR	DEATH RATE UNDER ONE YEAR PER 1000 BIRTHS
1905	141.4
1906	144.7
1907	135.7
1908	133.2
1909	126.8
1910	134.2
1911	120.9
1912	117.8
1913	110.6
1914	105.9
1915	101.9
1916	99.8
1917	97.4
1918	113.2
1919	88.5
1920	91.2
1921	75.9
1922	81.3
1923	78.1
1924	67.8
1925	73.1
1926	73.4
1927	64.7
1928	64.7
1929	62.0
1930	60.3
1931	54.8
1932	53.1
1933	51.9
1934	49.2

occurred first and most violently in New York and was followed by outbreaks in Washington, D.C., and Chicago, and in ten other cities¹ typhoid was "markedly excessive." In New York there were 913 cases reported between October 4 and February 14 as against a total expectancy of 204 cases for this period. Table XXXI shows the cases reported by weeks for the epidemic period and the cases reported for the corresponding weeks in the two previous years, in Washington, Chicago, and New York.

¹ Buffalo, Cincinnati, Grand Rapids, Jersey City, Memphis, Pittsburgh, Providence, Rochester, Scranton, and Yonkers.

TABLE XXXI. CASES OF TYPHOID FEVER REPORTED TO UNITED STATES PUBLIC HEALTH SERVICE BY CITY HEALTH DEPARTMENTS, BY WEEKS FROM OCTOBER 1, 1924, TO FEBRUARY 15, 1925, AND FOR THE CORRESPONDING WEEKS IN THE TWO PRECEDING YEARS¹

CITY	YEAR	OCTOBER				NOVEMBER				DECEMBER				JANUARY				FEBRUARY			
		4	11	18	25	1	8	15	22	29	6	13	20	27	3	10	17	24	31	7	14
Washington, D. C.	1922-1923	6	7	2	4	2	1	5	2	0	1	0	1	4	3	0	1	0	1	1	2
	1923-1924	1	6	1	2	5	2	1	3	5	1	1	1	0	3	1	1	1	0	0	0
	1924-1925	0	3	3	5	5	1	0	3	3	10	12	6	5	9	15	6	1	2	1	4
Chicago . . .	1922-1923	3	3	7	7	6	5	3	2	3	2	1	2	3	3	4	1	3	3	2	0
	1923-1924	5	7	5	10	11	20	25	33	77	71	41	32	14	20	21	14	16	10	5	8
	1924-1925	4	6	7	6	6	5	3	3	4	15	21	31	15	27	13	7	10	4	2	2
New York City	1922-1923	33	25	25	20	13	28	24	25	16	19	19	9	13	14	14	14	8	13	7	7
	1923-1924	34	21	32	19	16	17	15	18	10	18	20	7	6	4	21	21	9	11	16	7
	1924-1925	32	14	31	20	20	10	21	32	72	127	109	168	96	99	66	34	25	26	20	8

The experience was so unusual that an exhaustive epidemiological study of the several outbreaks was made, with the astonishing result that typhoid in these widely separated cities was traced to a common origin, namely raw shellfish supplied by a certain company operating in the vicinity of West Sayville, New York. While the exact source of the oysters was not determined, nevertheless it was certainly established that the typhoid bacilli were distributed over the country and finally reached the intestines of their human hosts by means of this vehicle.

This has led to more rigid supervision of the shellfish industry in general and of oysters in particular, so that through the inspection of the natural areas of production, the licensing of dealers, the certification of the health of handlers, and other measures the product is pretty well guaranteed up to the point where it reaches the local dealer. The exposure and handling of shucked shellfish in the market are still susceptible of improvement.

Oysters removed from polluted waters to clean sea water will in three weeks purify themselves of sewage bacteria.

¹ From *Supplement No. 50*, Public Health Report, United States Public Health Service, 1925.

Also both oysters and clams may be chlorinated. When placed in sea water containing chlorine, they will pump this water through their system and thus effect a disinfection of their intestinal tract as well as of the shell water. This is now practiced on a commercial scale.

Ice. Ice placed in drinking water or directly upon foods (such as butter or salads) is consumed in appreciable quantities. Natural ice is harvested from sources that would not be thought of as acceptable for water supplies. Water tends to purify itself as it crystallizes into ice, and many bacteria are destroyed by the destructive crushing between the forming crystals. Bacteria surviving this initial destruction gradually die out, and as natural ice is ordinarily stored for months before using, living typhoid bacteria are rarely present. The evidence for ice-borne typhoid is meager and unconvincing in the main. Recently an outbreak of thirty-seven cases was traced to an exceptionally low-quality snow ice, or ice that was formed from partly melted snow, which, in freezing, would not permit the natural purification discussed above.¹

Artificial, or manufactured, ice is at least as clean as the water it is made from; and since small quantities are required, the public drinking-water supply or a special spring water would naturally be used. Yet in making bacteriological tests of an artificial ice the author found astonishingly high counts. These were traced to a cloth filter which the manufacturers were using, presumably, to remove coarse dirt and sediment. Artificial ice does not have the safety factor found in storage.

Residual typhoid. Other food products may be infected accidentally in various ways. Food-handlers in the incipient stages of the disease, or with light, abortive cases, or as chronic carriers, may cause sporadic infections. Flies and possibly other vermin may transport sewage bacteria, on their feet or in their alimentary tracts, from the open drain or privy to the kitchen and dining room, depositing them on food which will be eaten without being subsequently heated. Vegetables, like lettuce and celery, may be washed in sewage-contaminated water.

¹ *American Journal of Public Health*, Vol. XIV (1924), No. 7, p. 574.

As water and milk supplies have become increasingly safe owing to modern methods of safeguarding them, the carrier has tended to assume a position of increasing importance in the spread of typhoid fever. From 2 to 4 per cent of typhoid convalescents may become carriers for an indefinite time. Such persons intermittently discharge typhoid organisms in their feces, and in rare instances in their urine, in enormous numbers. The bacilli usually localize in the gall bladder and the bile duct and not uncommonly cause gallstone formation. Because of the special risk that food-handlers of all sorts, including dairy employees, may infect the food, persons apprehended as carriers should be excluded from such occupations unless the condition can be cured. In institutions where the help is under close supervision, no one should be employed to prepare or serve food until at least two successive cultures from stools have proved negative for typhoid bacilli.

Owing to the advance of sanitary bacteriology the filth-borne diseases have declined in all civilized countries and communities. Epidemic typhoid is relatively rare today. The purification of water and milk has been the outstanding achievement. Slowly but surely the residual, or endemic, typhoid will recede as chronic carriers become fewer and as we increase our vigilance to keep filth out of food.

Food poisonings. There is a group of diseases, characterized by very sudden onset and acute gastrointestinal or nervous symptoms, that are called "food poisonings." These are all due to the growth or presence of bacteria in foods. They may have three general causes: (1) the presence of large numbers of living bacteria belonging to the paratyphoid-enteritidis group; (2) the presence of accumulated waste products resulting from bacterial putrefaction — the ptomaines; (3) the presence of specific secreted bacterial poisons, or toxins, such as the botulism toxin.

The acute infections by intermediate representatives of the colon-typhoid group are most common and most important. *Salmonella paratyphi*, *Sal. enteritidis*, *Sal. ærtrycke*, and *Sal. suipestifer* are the cultures most commonly isolated from the

stools of patients and from foods causing the outbreaks; but it seems probable that other related organisms, even *Esch. coli*, present in overwhelming numbers, may be responsible at times. The typical members of this group are animal pathogens, *paratyphosum* causing infections in man, and *enteritidis* and *suipestifer* being associated with diseased conditions in cattle and hogs respectively. Meat products are most commonly responsible for outbreaks of food poisoning. Infection of the food may take place before or after slaughter and during handling. The infected food does not necessarily give evidence of spoilage. Thorough cooking frees the food from infection. Careful protection and refrigeration of cooked food, especially chopped-meat products, and of foods taken from cans are essential safeguards.

The opinions regarding the importance of true *ptomaine poisoning* have undergone rapid revision during the last few years. Ptomaines are decomposition products derived from protein foods by bacterial action. Experimental laboratory work and the study of outbreaks of food poisoning have revealed the overwhelming importance of the acute infections just discussed as compared with true ptomaine poisoning.

Botulism has assumed prominence of late owing to a number of outbreaks that have occurred in the United States, particularly during and since the war years. Botulism is a true poisoning caused by an extremely potent, soluble, secreted exotoxin produced by *Clostridium botulinum* growing in food. This toxin is not destroyed by the gastrointestinal secretions, as is the case with all other known true bacterial toxins. The organism is not a pathogen, but is a spore-forming anaërobic saprophyte having a wide distribution in nature. It grows readily in foods of either animal or vegetable origin; and, being a spore-former, it resists ordinary cooking temperatures. Its anaërobic habit permits it to grow in canned products, either homemade or commercially prepared.

The symptoms of botulism are paralytic in nature. The botulinus toxin is the most potent of any known, fatal cases being recorded in which the person has merely tasted the food

and not even swallowed it! In white mice dilutions so great that theoretical estimates give as little as one molecule to the dose prove fatal. Hence the previous warnings about tasting canned foods that one is suspicious of, to see if they are safe.

The botulism toxin is destroyed by boiling. Though the spores of the organism are not destroyed by such heat, nevertheless the food is safe after boiling, for it has been noted that the organism does not multiply or produce toxin in the intestine.

In certain outbreaks of food poisoning staphylococci have been isolated from the responsible food. Soluble toxic products have been recovered from cultures of these organisms.

Cooking food thoroughly is the principal safeguard against food poisoning. It does not destroy the chemical bodies known as ptomaines; but it is doubtful if they are important, and foods containing them would usually be detected as spoiled.

CHAPTER XXI

THE PUBLIC-HEALTH LABORATORY

The public-health laboratory is an indispensable adjunct to the proper promotion of health and to the practice of modern medicine. As our knowledge of the specific causes of the various communicable diseases has grown, the services of the laboratory have expanded until today, in all but five or six of the important reportable diseases, the laboratory serves as a direct aid in diagnosis or provides curative or prophylactic products. The proper control of diseases dangerous to the public health involves not only the correct diagnosis of the case of contagious disease, but demands also that, whenever possible, release from isolation shall be sanctioned only when the disease microorganisms have entirely disappeared from the patient. In diphtheria the organisms from the nose and throat can be identified, and in this disease it is common to release the patient only when two successive examinations show the cultures to be negative. The discovery of carriers — that is, well persons who harbor pathogenic germs, as in typhoid fever — is a very important matter in prevention. Carriers can be detected only with the aid of the laboratory.

Sanitary analyses of water, milk, shellfish, and other foods have already been discussed. Public-health laboratories, or other official or private laboratories, are essential for the routine tests that assure communities safe and wholesome food commodities.

The laboratory divisions of many of our state departments of health and some of our large city departments engage in the preparation and dispensing of biological products which are used for preventive or curative purposes. The proper manufacture of these sera and vaccines requires the highest technical skill and supervision. The use of these products

gives the most reliable and specific results known to medicine and has led to very remarkable reductions in the prevalence and in the fatality of some of our most dreaded diseases.

The importance of laboratories in the field of public health is reflected in their rapid growth in our cities and states. The first public-health laboratory for bacteriological diagnosis was established in New York City in 1893. A study of the hundred largest cities in the United States made by the United States Public Health Service and a committee of the American Public Health Association in 1923 revealed the fact that all the cities enjoyed the benefit of laboratory service and that eighty-nine of them were provided with special laboratory facilities that received official support. The other eleven cities either were located so that they could use the state or county laboratories or else entered into a contract with local private laboratories. The various attempts that have been made to appraise different public-health activities allow laboratories from 5 per cent to 10 per cent of the total. The appraisal plan approved by the American Public Health Association allows laboratories a possible 70 out of 1000 points. The function of the laboratories in the various fields mentioned will grow as our knowledge of the biology of the communicable diseases grows. The existing services are not taken full advantage of today, and it is most regrettable that there are still many persons who not only ignore but actively resist the application of these most remarkable and beneficent of all discoveries in conserving human life.

Hospital laboratories. Laboratories that serve particular hospitals apply the same general principles of bacteriology to the diagnosis of disease, but their point of view is somewhat different. Hospitals deal with the sick, and the study of specimens is primarily for the benefit of the patient and not for the public health. That does not mean that the hospitals, in dealing with communicable diseases, do not play an important part in the prevention of disease, but only that this aspect, so far as the laboratory is concerned, is incidental. Many specimens coming to the hospital technician require chemical

and histological study to detect abnormal metabolism or pathological conditions in the tissues.

Public-health-laboratory diagnosis. The laboratory may contribute to the diagnosis or prevention of twenty-three out of twenty-eight of the communicable diseases that are commonly made reportable. The tests are extremely delicate and highly specific. The results are not perfect, any more than are results in other fields of human endeavor. Errors arise owing to various causes, including faulty collection and shipment of specimens and also lack of technical skill in manipulation and observation in the laboratory. Each case should be handled separately, and the physician will use the laboratory evidence furnished him as only a part of the whole diagnostic picture.

The principles involved in the laboratory technique may be conveniently classified as follows: (1) The direct microscopic examination of the original specimens, or cultures prepared from them. (2) Animal inoculations to detect the presence of living virulent organisms. (3) The observation of specific reactions occurring between antibodies present in the blood of the patient (or in animals that have been specifically immunized) and antigens. (Antigens are usually the microbes themselves or their products, but in the case of syphilis a special chemical preparation is used which has the properties of an antigen.) (4) Combinations of any of these general methods.

Direct microscopic examination is evidently the most satisfactory and expeditious method of study, where it is applicable. It depends upon the presence of the microorganism in sufficient numbers at some accessible site, and upon the organism's possessing some highly characteristic morphological or staining properties. The diseases commonly diagnosed by this method include pulmonary tuberculosis, diphtheria, gonorrhea, meningitis, Vincent's angina, and malaria. Rabies in dogs is likewise usually diagnosed by the microscopic method.

Tubercle bacilli are found in the infected organs of animals and humans, and in pulmonary tuberculosis these organisms

are discharged with the sputum. They may be found in the intestinal wastes of those suffering from intestinal infection or of those who have swallowed the organisms with their sputum. They are eliminated with the urine in kidney infection and are at times present in small numbers in the blood.

From the standpoint of microscopic diagnosis the presence of typical tubercle germs in the sputum is most significant. Their detection depends primarily upon characteristic staining properties and upon morphology. The *Mycobacterium tuberculosis* of human origin, as it occurs in sputum, is a delicate, slender, slightly curved rod, occurring singly, but more often in pairs or clumps, the long axes lying more or less parallel (see Frontispiece). In culture the cells are sometimes club-shaped or branched—a fact which seems to ally them with the Actinomycetales. The tubercle bacillus stains poorly or not at all with the ordinary dyes; but once a powerful dye like carbol-fuchsin, aided by the mordant and by heat, has penetrated the waxy envelope surrounding the cell, it retains the stain tenaciously even when treated with a powerful decolorizing agent, such as alcohol containing a mineral acid. Organisms possessing these special staining properties are called acid-fast or acid-proof organisms.

Sputum from a suspected patient, brought to the laboratory, is smeared liberally over a clean glass slide; and after drying and fixing, it is flooded with Ziehl-Neelson's carbol-fuchsin stain, and is then steamed for from one to five minutes. It is decolorized with acid alcohol (2 per cent HCl in 80 per cent to 95 per cent ethyl alcohol) until all the visible color has been washed out, and is then counterstained with methylene blue, to give a contrast color as a background. The pink bacilli stand out sharply in a blue field. The diagnosis may be simple if the bacilli are numerous, or it may require a patient, exhaustive search before a final decision can be conscientiously given.

Some laboratories first treat the sputum with a mixture of sodium hypochlorite and sodium hydroxide (antiformin), which dissolves much of the mucus and foreign bacteria,

leaving the tubercle bacilli intact. After this treatment the bacteria may be concentrated by centrifugation and stained in the manner previously described.

The leprosy bacillus (*Myco. lepræ*) is an acid-fast organism, and the laboratory diagnosis is made from swabs taken from the nose or leprosy lesions and staining and examining in the same manner as for tuberculosis.

The laboratory diagnosis of *diphtheria* is the most universal and perhaps the most important test that is made in public-health laboratories. It is used not only to confirm the bedside diagnosis in suspicious cases but also for the release of patients from isolation and for the detection of carriers. The preparation of the specimen for examination is very simple, but the proper interpretation of the findings is difficult and depends upon acute observation and long experience. In skillful hands, however, a very high proportion of the findings will be correct.

The specimen is taken by the physician upon two sterile cotton applicators, one from the throat and another from the nose. The applicators are carried or sent to the laboratory immediately, and a preliminary study is made directly from the cotton swabs. The swabs are then rubbed on blood-serum media and incubated. Then the cultures are examined microscopically at intervals up to twenty-four hours.

The examination consists in making smears and in staining by one of several special methods — usually with Löffler's methylene blue. There are on the swab and in the culture a variety of organisms which occur in the nose and throat, and the diphtheria bacilli must be differentiated from these other bacteria.

The *Corynebacterium diphtheriæ* appears as a slender rod, straight or bent, often club-shaped, dumb-bell-shaped, or spindle-shaped (see Frontispiece). The bacteria vary markedly in shape and in size. Two or three cells together, making V-shaped or Y-shaped patterns, are frequently observed. The cells stain irregularly, showing deeply staining granules or bars, usually located in the swollen portions. These highly refractive granules are the most characteristic single feature.

Occasionally, branching structures are observed. This characteristic is the basis for the classification of diphtheria with the Actinomycetales.

Nonvirulent bacilli that fail to produce acid from dextrose, and that morphologically are shorter, plumper, and without granules, are sometimes encountered in healthy throats. This form is represented by the Hoffmann bacillus, a pseudodiphtheria bacillus. The possibility of modification of virulence in these forms has frequently been discussed, but positive proof of this seems still to be lacking.

Nowhere in diagnosis do the morphological differences between organisms play such an important part as in distinguishing true diphtheria bacilli from the pseudo forms. Young cultures grown on Löffler's blood serum assist greatly in distinguishing between the two, and a careful study of the morphological characters at this stage makes the diagnosis certain when in the hands of an experienced person. Avirulent diphtheria bacilli are probably not as common as some would have us believe. The fault lies rather with the inability of an inexperienced observer to distinguish between diphtheroids and the true diphtheria bacillus. In cases of doubt, or when the organisms persist for a long time in the throat, virulence tests, made by animal inoculation, are desirable.

Gonorrhea is a specific infection caused by a Gram-negative diplococcus, *Neisseria gonorrhææ*. Infection usually occurs in the urinogenital tract, causing inflammation, with the production of pus. Gonorrheal ophthalmia, or infection of the delicate membranes of the eye, may occur in infancy or early childhood and is a cause of preventable blindness. The specimens usually sent to the laboratory for examination consist of smears made upon microscopic slides directly from the inflamed area, usually from the urethra or from the conjunctiva. The slide is treated by the Gram staining method. The typical gonococci are completely decolorized in the alcohol and take the counterstain. It is well to control the staining reagents occasionally by staining known Gram-positive and Gram-negative organisms.

A positive report depends upon the presence of characteristic coffee-bean-shaped or biscuit-shaped cocci, occurring in pairs, the flattened sides adjacent (see Frontispiece). Many of the cocci are seen within the pus cells, or leucocytes, and their intracellular position is an important element in the diagnosis. Gram-negative cocci are not uncommon in the discharges from the mucous membranes which are the site of infection with gonococci. Hence the staining, morphology, grouping, and intracellular position of the organisms all form an essential part of the diagnostic picture.

Epidemic meningitis is caused by a Gram-negative diplococcus (*Neisseria intracellularis*), which is intracellular and appears very much like the gonococcus. The specimen examined for meningitis consists of fluid obtained by puncture from the spinal canal of a suspected case. There is little danger, therefore, of confusing it with gonorrhea, although the gonococcus may rarely cause inflammation of the meninges. *Neisseria intracellularis*, observed in the exudate from the meninges in paired or tetrad form, occurs almost always within the polymorphonuclear white blood cells. Sometimes these cells are crowded with cocci. Individual cocci often vary quite strikingly in size. No organisms are found naturally in the spinal fluid, so the diagnosis is practically certain.

The supreme importance of an early and correct diagnosis of meningitis lies in the fact that there is a highly reliable and specific antiserum for the treatment of this type of infection. Meningitis may be caused by any one of several organisms, but this serum prophylaxis is applicable only when the meningococcus is the causative agent.

Other diseases may be diagnosed by direct microscopic observation, such as Vincent's angina (trench mouth), caused by a fusiform bacillus in conjunction with a spirochæte (see Frontispiece); syphilis, by the direct examination of material from suspected lesions with dark-field illumination, which reveals the delicate, nonrefractile, actively motile, corkscrew-shaped *Treponema pallidum*; anthrax, recognized by its large dimensions, chain formation, and spores.

Not only specific bacteria but also some parasitic protozoa, or pathologic conditions in certain tissues, may be identified by microscopic observation of stained preparations. Malaria is diagnosed from a drop of blood taken from the suspected patient and smeared on a microscopic slide. In the human body the malarial organism passes through successive asexual cycles within the red blood cells. The parasites are sought within the corpuscles, appearing in various stages of development as ringlike bodies near the margin of the cell or as amoeboid or rounded organisms occupying a large portion of the red cell. Granules of pigment appear, scattered irregularly or more or less massed within the parasite. At the later stages of development segmentation occurs, the parasites finally rupturing the cell and appearing as spheroidal bodies which proceed to attach themselves to red corpuscles and to repeat this cycle. The paroxysms of chill and fever in the patient are coincident with this maturation of the parasites in the blood.

Rabies in dogs should always be confirmed by the laboratory. The dog's head is sent to the laboratory, and smears are made upon microscopic slides from certain definite regions of the center of the brain. The stained slides from positive heads reveal characteristic round or oval inclusions within the large nerve cells (see Frontispiece). These are known as Negri bodies, named after their discoverer, and probably represent one stage in the life cycle of an animal parasite or are agglomerations or colonies of a minute virus, although there is a possibility that they are the result of cell degeneration.

Animal inoculation. It is often necessary to inoculate animals in the laboratory in order to make correct diagnoses or to determine the virulence of an organism. The animals most commonly used are guinea pigs, rabbits, and white mice. Work with animals should be carried on in the most humane way possible, by using anæsthetics during painful operations and by killing animals promptly and humanely as soon as the desired results have been obtained.

Animals are used to obtain the growth of organisms from suspected material when they grow with difficulty, or not at

all, upon any known artificial culture medium. It is necessary at times to use animals to obtain a pure culture of an organism. With diphtheria, in the case of a healthy carrier or when the bacteria persist in the throat of a convalesced patient, the virulence, or toxicity, of the organism may be determined by the inoculation of a guinea pig.

Animals are commonly inoculated by the use of a hypodermic needle. Various paths of inoculation may be used, such as underneath the skin (subcutaneous); into one of the body cavities, for example the peritoneal cavity (intraperitoneal); or into a vein (intravenous).

In tuberculosis the specimens may contain so few tubercle bacilli that they cannot be detected by the microscopic method, although the history and clinical picture are strongly suspicious. Moreover, in tuberculosis of the kidney another acid-fast organism, notably *Mycobact. smegmatis*, may be present to confuse the diagnosis. In such cases the injection of some of the suspected material subcutaneously into a guinea pig will produce tuberculosis within four to six weeks. The animal is then chloroformed, and, upon autopsy, characteristic lesions are found in the spleen, liver, or other glands, which can be confirmed by the microscopic examination.

When rabid animals are killed too early in the course of the disease, or when the brain is badly decomposed, the Negri bodies fail to show. The animal may have bitten someone and may have been in an infectious state, making it imperative to obtain a correct diagnosis. If there is real suspicion of rabies, the person who has been bitten or even licked by the dog should commence the Pasteur treatment at once and not wait for the results of animal inoculation. Rabbits inoculated subdurally with infected brain material develop the characteristic symptoms of rabies ordinarily within four weeks.

The examples cited will suffice to show the importance of animals in diagnostic laboratory work. In the tests to be discussed next, — namely, reactions between microbes, or their extracts, and immunity substances, — the use of animals is indispensable, as will be seen.

Antibody-antigen reactions. The invasion of the body by microbes always results in a defensive reaction on the part of the host. This reaction may be feeble and insufficient to eradicate the invader, or it may be vigorous, resulting in complete recovery and, in most cases, in a continuing heightened resistance which we call immunity. In many diseases this defensive reaction is accompanied by the production of chemical substances, or antibodies, which can be demonstrated by laboratory tests. Gruber and Durham (1896) were the first to study the reactions between such specific substances and infecting bacteria in the case of clumping substances, or agglutinins. In the same year Widal reported the classic test for typhoid fever which bears his name.

The Widal test, or agglutination test for typhoid, is performed by mixing the diluted blood of a suspected patient with a known culture of typhoid and observing the characteristic clumping that will occur if the disease exists. A single drop of blood is sufficient for the test. Since the agglutinin will withstand drying for an appreciable time, the specimen may be taken at the bedside, dried on a slide or on a special aluminum plate provided for the purpose, and sent to the laboratory through the mail. The dried blood is worked up with a drop of physiologic salt solution and is then diluted to one part in ten by mixing one loop with nine loops of the salt solution. The final dilutions used for the test — usually 1 : 20 and 1 : 40 — are made by mixing equal parts of the diluted blood and the suspension of a young typhoid culture. This is mounted in a hanging drop and examined under the microscope for the gradual immobilization and clumping of the bacilli. In typically positive cases the reaction should be complete in twenty minutes. A negative reaction does not necessarily mean that the patient has not typhoid fever, for during the first few days of the disease agglutinins may be absent and do not reach their maximum concentration for ten to fourteen days following the onset. In clinically doubtful cases the laboratory test should be repeated. If the specimen permits, a macroscopic test may be made by appropriately diluting

the blood and typhoid culture in the test tube and observing for the appearance of a flaky precipitate.

Typhoid-bacillus carriers will give a positive Widal test in the majority of cases. In the search for carriers in epidemiological work the test may be used as a sort of screen to separate suspected from unsuspected persons. The examination of the stools (intestinal wastes) for the actual organisms in those giving positive Widal's will usually apprehend the carrier. This practice saves time and an immense amount of work when compared with stool examinations for all possible responsible persons in a typhoid outbreak caused by carriers.

Agglutinins persist for some time in persons vaccinated against typhoid, and the history of vaccination, as well as of previous attacks, should be obtained.

Agglutinins are formed during the onset and course of many diseases. They are always highly specific and may be applied not only for diagnosis but also for identifying different species of bacteria that are culturally and morphologically similar, and by special absorption methods they may be used for differentiating distinct races or types within a species.

The most important application of the typing of bacteria is made in the diagnosis of *pneumonia*. To know the type or group of pneumococcus causing infection is to know the possibility of specific serum treatment. This test depends upon the presence of both specific agglutinins and precipitins. Precipitins are specific substances appearing in the blood early in the course of an infection. They are formed not only in man but in other animals injected with bacteria such as pneumococci. For the typing of pneumonia in the laboratory there must be provided the serum from horses that have been immunized for Types I, II, and III respectively. The specimen of sputum coming from the patient may be specially treated and used directly with the horse sera, or it may be cultured, or inoculated into a mouse. In any of these methods the antigen is ready to add to the type sera, properly diluted, in from five to eight hours. After one hour's incubation the three tubes are carefully examined for a white, cloudy precipitate con-

sisting of the agglutinated organisms or the precipitated antigen. The tube in which the precipitate appears is positive and indicates the type of infecting organism. If no precipitate occurs, and yet pneumococci are present as viewed under the microscope, then obviously it must be a Group IV organism. A modification of this test by agglutination of the organisms by one of the three known sera on a microscope slide may be used.

Another rapid and reliable test for pneumococcus typing depends upon the swelling ("Quellung") of the capsule of the organism in the presence of specific immune serum. This is known as Neufeld's "Quellung" test and is performed by mixing the sputum coming directly from the patient with an equal amount of the known Types I, II, and III immune sera and examining directly under the microscope. The reaction is completed in from two to three minutes. If differentiation between Types IV-XXXII (Group IV) is desired, immune serum for each of them must be available for separate tests.

Gonococci, meningococci, and other organisms may be typed by similar agglutination or precipitation techniques.

The complement-fixation test (Bordet-Gengou phenomenon) has many important and practical applications, not only in the diagnosis of various diseases but also, because of its specific nature and delicacy, for the forensic determination of foreign proteins in very minute amounts. Its most important and universal use, however, is in the diagnosis of syphilis by the so-called Wassermann reaction. Syphilis is difficult to diagnose in its early stages; but it is supremely important to detect its presence, both for the favorable chances of cure of the patient and for the protection of the public health. This test is specific and reliable.

It has been noted that the inoculation of foreign albuminous substances (antigens) into the animal body, as well as the invasion by microbes, results in the production of antibodies. The antibodies that produce lysis, or the dissolving of cells, are known as amboceptors, and their action depends upon the presence of a nonspecific complementary substance which is

always present in all fresh blood serum. The complement is not adsorbed, or fixed, by antigen or amboceptors alone, but only by antigen and amboceptor united. However, antigen and specific amboceptor will combine, or antigen will adsorb the antibody in the absence of complement.

The Wassermann test involves many difficult theoretical considerations; but the test itself, once the reagents are at hand, is easily comprehended. Five reagents enter into the test: four are known, and the fifth, the patient's serum, is the unknown, to be determined. The reagents are (1) *antigen*, usually consisting of an alcoholic extract of cholesterinized heart muscle; (2) *complement*, from fresh guinea-pig serum; (3) *hæmolytic serum*, prepared by inoculating rabbits at intervals with washed red blood cells of sheep (the serum is heated, or inactivated, to destroy the complement naturally present); (4) *red blood cells* from sheep, thoroughly washed, and mixed with physiologic salt solution to make a 5 per cent suspension; (5) the *patient's serum*, to be tested. This is usually obtained from blood drawn from the arm vein. It is heated to destroy the complement.

The test is performed by adding definite amounts of antigen, patient's serum, and complement to a test tube and placing it in a water bath at 37° C. for a definite time. Then the hæmolytic system, consisting of the red cells and the heated rabbit serum which have been previously mixed and incubated, is added, and the whole mixture is incubated again. If the red cells now remain whole, the contents of the tube appearing a cloudy red, it indicates a positive test: the suspected serum came from a syphilitic patient. This is due to the fact that all the complement was fixed by the antigen-antibody combination in the first incubation, leaving none to activate the hæmolysis of the red cells. If the red cells dissolve and the tube shows a partial or complete clear-red color, the test is negative. The patient's serum in this case does not contain the necessary antibody for the antigen, and therefore the complement remains free to combine with the hæmolytic system and to cause hæmolysis.

Various so-called precipitation tests have been developed to supplement, and in some cases to displace, the Wassermann test for syphilis. These tests all depend upon a colloidal reaction between lipoidal tissue extracts and the immune bodies in the patient's serum in the presence of salt solution, which causes an opaque, finely flaky precipitate to appear. Varying amounts of syphilitic antibody require different amounts of the precipitation extract reagent; so at least three quantitative tests are ordinarily set up. The best known of these tests in this country are the Kahn test and the Hinton test. The latter test depends upon the presence of glycerin, which, it is claimed, gives a sharper, more easily read precipitate.

The preparation of biological products. In addition to the work in public-health and hospital laboratories just described, the bacteriologist engages also in the preparation of biological products which may be used for either the treatment or the prevention of many of the infectious diseases. These products include the most specific and reliable weapons at our disposal in combating infectious disease, both for treatment and for prevention. The field of application is being rapidly extended, and the next few years may see further spectacular discoveries that will lead to the control of some of our remaining enemies. The following list includes only those diseases in which the use of such products has been demonstrated by extensive experience to be reliable.

Diseases treated by biologic products

By antisera

Diphtheria

Tetanus

Scarlet fever

Pneumonia, Types I and II

Meningitis, epidemic cerebrospinal

Plague

Gas gangrene

By placental extracts

Measles

By vaccines

Whooping cough

Staphylococcus infections

Streptococcus infections

By bacteriophage

Staphylococcus infections

Diseases which can be actively immunized against

By means of vaccines

Smallpox

Rabies

Typhoid and the paratyphoids

Whooping cough

Plague

Cholera

Tuberculosis

Yellow fever

Rocky Mountain spotted fever

By means of other antigens

Diphtheria

Scarlet fever

Hay fever

The use of biological agents to produce immunity against specific infectious diseases had its beginnings centuries ago with the Chinese, who used the dry crusts from smallpox scabs, which they inserted in the noses of healthy people in order to induce smallpox. The disease resulting from this artificial inoculation was ordinarily milder and less fatal than smallpox acquired naturally. This practice spread through Europe and into America during the eighteenth century.

Jenner's epoch-making discovery (1796) of the use of cowpox virus to induce immunity against smallpox established a new principle; namely, that the body will react to a mild, though otherwise identical, virus so as to acquire the same degree of resistance as recovery from the disease itself would give. This principle was not extended until the era of modern bacteriology had developed a knowledge of specific germs as the cause of specific diseases and until these diseases had been studied experimentally by the use of animals.

The manufacture of these products, either by official or by private laboratories, can be performed only after a license has

been obtained from the Secretary of the Treasury upon recommendation of the Surgeon-General of the United States Public Health Service. The establishments are frequently inspected, and samples of all products are examined from time to time by the Hygienic Laboratory at Washington to determine their reliability and potency.

The following description of the preparation of some of these products will serve to show the general technique and care that are used in their manufacture.

Vaccine virus for smallpox is obtained from healthy calves that are artificially inoculated with known specific virus of cowpox, or vaccinia. The seed virus is obtained in various ways, usually by passage through rabbits. This virus is inoculated into a young healthy female calf that is proved free from tuberculosis and from all other detectable diseases. The hair is clipped from the body, and the animal is thoroughly cleaned. Just before vaccination the abdominal surfaces are shaved, then washed with soap and water and with alcohol and water, and finally dried with a sterile towel. These surfaces are scarified, and the seed virus is introduced. When the vesicles have developed to a certain stage (usually on the fifth or sixth day), the calf is anæsthetized and killed, the crusts are picked off, and the pulpy contents of the vesicles are removed. They are mixed with glycerin,¹ thoroughly ground, placed in sealed bottles, and stored, to ripen for a time. The finished product is tested for purity by planting it, aërobically and anaërobically, in culture media and by animal inoculation. It is also diluted, and tested for potency by vaccinating rabbits. The vaccine is put up in containers for shipping, each one clearly marked by number, and stamped with the date of issue and the date beyond which it will be unreliable for use. The vaccine must be kept continuously cold or it will rapidly deteriorate.

The fixed virus of rabies first used by Pasteur in the preven-

¹ A mixture containing 50 per cent glycerin and 1 per cent carbolic acid will prove germicidal to bacteria, including spore-formers, but will not injure the vaccine virus. Brilliant green dye, added to the glycerin-carbolic mixture to make a dilution of 1:10,000, enhances and hastens this selective purification.

tion of this disease is still used in many parts of the world. Fixed virus is a virus of stabilized virulence obtained by successive passage through rabbits until it uniformly causes death in this animal within six or seven days. By drying for times varying from three to eight days the spinal cord derived from rabbits artificially infected with fixed virus, all grades of virulence can be obtained. Starting with completely avirulent virus, the potency is gradually stepped up until at the end of the twenty-first day of treatment a practically fully virulent dose is given.

In this country the rabies virus has been modified or killed in various ways, such as by drying and freezing or by treatment with carbolic acid or with formaldehyde. Uniform doses of these killed viruses are administered daily for fourteen days. The reactions in the patient are less marked, and the protection seems to be as great as that obtained by the Pasteur method.

Dogs are now being protected for periods of six months to a year by a single large injection of phenol-killed or chloroform-killed rabies virus.

The vaccine used for immunizing infants against *tuberculosis*, usually called B. C. G. vaccine (*Bacille Calmette-Guérin*), is a culture of the bovine strain of tubercle bacillus which has been reduced and stabilized in virulence by long cultivation in artificial media containing bile salts. The opponents of its use believe that it may possibly dissociate into smooth, virulent strains; but in spite of one or two sad experiences where the true cause seems doubtful, the practice of using this vaccine in infants especially exposed to tuberculosis by being born into families where active cases exist has become quite general in many countries of the world with what appear to be strikingly favorable results.

Bacterial vaccines (more appropriately called bacterins) are all prepared in a similar manner. Vaccination against typhoid is the most universal and thoroughly tested practice.

A known pure, smooth, motile culture of the typhoid organism is grown on standard agar media for twenty-four hours. It is then washed off in physiologic salt solution and heated

for one hour at 53°-56° C. The heated suspension is tested for sterility by planting aërobically and anaërobically and by animal inoculation. It is standardized by counting the number of bacteria contained in a cubic millimeter and is then diluted so that one cubic centimeter contains approximately 1,000,000,000 cells. A preservative is added, and it is put up in bottles or in ampoules containing a specified dose. It is usually administered in three injections, spaced a week apart: the first dose, of 500,000,000 killed bacilli, and the second and third doses, of 1,000,000,000 each. Ordinarily the reactions to these inoculations are mild and local.

Polyvalent vaccines may be prepared which contain the killed suspensions of two or more types of bacteria. Thus, cultures of the paratyphoid groups may be combined with typhoid, and a single series of inoculations will result in immunity to the several infections.

Autogenous vaccines are prepared from cultures recovered directly from the infected site in the patient to be treated. This assures the use of the identical antigen causing the disease; and local and chronic infections often yield to such vaccine treatment. With all bacterial vaccines the choice of the strain or strains for use should be taken into consideration. The specific antigenic properties possessed by smooth as compared with rough cultures, and the special antigenic properties of the flagella, make a careful selection of the particular culture to be used an important matter.

Diphtheria toxoid, now generally being used for active immunization of children against this disease, consists of a bacteria-free filtrate of a broth culture of toxogenic strains of diphtheria bacilli. This is titrated for its toxic potency, treated with formaldehyde (0.4 per cent), and incubated at 37° C. until it is completely detoxified. Before distribution it is carefully tested for sterility, and by animal inoculation to assure that no active toxin remains, and is accurately standardized.

Bacteriophage has given contradictory results in the hands of different users. Filtrates containing phage also contain the dissolved products of bacteria and constituents of the media

in which they have been grown. These, acting as vaccines, may explain the favorable results found in some cases. Opinion is pretty well united that in local staphylococcus infections and in cystitis bacteriophage is distinctly helpful. It is a field for further study, with much promise when the mechanism involved is better understood.

Antitoxins and *antisera* are of value chiefly in the treatment of disease, though they also have value in emergencies in that they give a temporary passive immunity in known cases of exposure of non-immunes. The manufacture of these antibodies must obviously be carried out by the use of animals. The horse, on account of its large size, its docility, its freedom from diseases transmissible to man, and, finally, its ability to produce the desired antibodies in high concentration, is ordinarily selected as the animal to be used. Some laboratories prefer goats as animals to use for the manufacture of antisera.

The production of diphtheria antitoxin, the first and most universally used serum, may be taken to illustrate the preparation of these products. The soluble toxin from a highly toxic strain of diphtheria is freed from the organisms by filtration. Healthy horses that have been tested in every known way for disease are injected every five to seven days, at first with very small doses of the toxin. Gradually the doses are increased in size until relatively enormous amounts of this poison are tolerated. Different animals vary a good deal in their tolerance to this treatment and also in their ability to give a high yield of antitoxin in their blood.

When the antitoxin content of the blood has reached a sufficient potency, the animals are bled from the jugular vein into sterile jars, all aseptic precautions being taken. They are scarcely annoyed by the withdrawal of from five to eight liters of blood, and the bleedings may be repeated as often as once a month without injury.

Upon standing, the serum separates from the clot and is drawn off aseptically.¹ It is filtered through a Berkefeld filter

¹ Another method permits the use of sodium citrate or potassium oxalate to prevent clotting.

and is concentrated by precipitating the pseudoglobulins (which contain most of the antitoxin principle) with ammonium sulphate. The albumins remain in solution and are removed. The precipitate is dissolved in saturated sodium chloride, and then the salts are removed by dialyzing in running water. By thus concentrating the antitoxin fraction of the serum, much smaller amounts may be used in treatment, and a large part of the foreign blood proteins are removed, thus reducing the danger of untoward reactions and the development of rashes following injection.

Finally the antitoxin must be standardized by determining its strength, or the number of combining antitoxin units present in a cubic centimeter. The Standard Unit of antitoxin is kept by the Hygienic Laboratory at Washington, and all manufacturing laboratories are provided with this unit, upon request, as a common starting-point to determine the amount of antitoxin in their unknown serum. The therapeutic unit is determined by accurate quantitative tests that are based upon the amount of antitoxin necessary to protect guinea pigs of a certain size from one hundred times the smallest fatal dose of diphtheria toxin. From this it can be readily calculated how many units of antitoxin there are in one cubic centimeter.

The standardized antitoxin is put up in bottles or ampoules clearly marked with the number of units contained.

The dose [to be used depends upon the stage and severity of the disease and upon the age of the patient. Any amount from 3000 to 20,000 units may be advisable. Serum commonly contains from 500 to 700 units per cubic centimeter and sometimes contains double these amounts.

APPENDIX A

IMPORTANT DATES IN BACTERIOLOGY

MICROSCOPE

- 1590 JANSSEN, HANS and ZACHARIAS. Invent compound microscope
- 1760-1776 MARTIN, BENJAMIN. Improves microscopes
- 1844 DOLLAND. Originates immersion lens
- 1870 ABBÉ, ERNST. Invents substage condenser
- 1903 SIEDENTOFF, H., and ZSIGMONDY, R. A. Invent dark-field microscope
- 1925 BARNARD, J. E. Discovers ultra-violet photography

GERM THEORY OF DISEASE

- 1546 FRACASTORO, GIROLAMO. Formulates theory of infection ("seminal contagionum")
- 1658 KIRCHER, ATHANASIOS. Makes first use of microscope to investigate disease. Announces definite statement of germ theory of disease ("contagium animatum")
- 1665 HOOKE, ROBERT. Originates advanced microscopy; describes cell structure of organisms
- 1668 REDI, FRANCESCO. Is the first to make experimental refutation of prevalent theory of spontaneous generation
- 1683 LEEUWENHOEK, ANTON VAN. Discovers and describes bacteria
- 1718 MONTAGU, LADY MARY W. Introduces inoculation against smallpox into Western world; has son inoculated
- 1762 PLENCIZ, MARCUS VON. Modern concept of germ theory of disease (each germ a specific cause)
- 1775 SPALLANZANI, LAZARO. Makes experimental refutation of theory of spontaneous generation in "infusions"
- 1786 MÜLLER, O. F. Is the first to attempt classification of bacteria
- 1796 JENNER, EDWARD. Discovers vaccination against smallpox
- 1800 WATERHOUSE, BENJAMIN. Introduces vaccination against smallpox into America
- 1838 EHRENBERG, C. G. Publishes systematic classification of bacteria; introduces some of the names now used
- 1839 SCHÖNLEIN, JOHANN. Discovers the mold *Achorion schönleinii* as parasitic cause of favus
- 1843 HOLMES, OLIVER W. Proves contagiousness of puerperal fever
- 1848 PASTEUR, LOUIS (1822-1895). Discovers molecular dissymmetry

- 1854 SCHRÖDER and DUSCH, V. Introduce use of cotton plugs
1856 PASTEUR, LOUIS. Establishes microbic cause of fermentations
1863 PASTEUR, LOUIS. Discovers cause of "disease" of wine; pasteurization of wine
1865 PASTEUR, LOUIS. Discovers cause and prevention of silkworm disease
1865 DAVAINÉ, CASIMIR. Discovers anthrax bacillus; inoculates this disease artificially
1865 VILLEMEN, J. A. Demonstrates infectiousness of tuberculosis
1867 LISTER, JOSEPH. Introduces antiseptic surgery
1871 WEIGERT, CARL. Is the first to stain bacteria with picrocarmine
1871-1874 HANSEN, ARMAUER. Establishes causal relation of bacteria of leprosy
1876 KOCH, ROBERT. Makes microscopic studies of bacteria. Describes spores and life cycle of *B. anthracis*. Proves cause of anthrax
1877 KOCH, ROBERT. Makes dried films, and stains bacteria with anilin dyes
1877 PASTEUR, LOUIS. Discovers cause of anthrax and chicken cholera
1877 LISTER, JOSEPH. Prepares pure cultures by dilution method
1879 NEISSER, ALBERT. Identifies organism causing gonorrhea
1880 EBERTH, KARL JOSEPH. Isolates typhoid organism
1880 LAVERAN, ALPHONSE. Discovers plasmodium causing malaria
1880 PASTEUR, LOUIS. Discovers attenuation of cultures; introduces protective inoculation against chicken cholera
1881 PASTEUR, LOUIS. Discovers protective inoculation against anthrax
1881 KOCH, ROBERT. Uses solid media in obtaining pure cultures
1882 KOCH, ROBERT. Discovers tubercle bacillus. Formulates Koch's "postulates"
1883 KLEBS, EDWIN. Discovers diphtheria bacillus
1884 LÖFFLER, FREDERICK. Establishes cause of diphtheria
1884 NICOLAÏER, ARTHUR. Identifies organism causing tetanus
1884 METCHNIKOFF, ÉLIE. Discovers phagocytosis
1885 PASTEUR, LOUIS. Discovers preventive vaccination against rabies
1886 ESCHERICH, THEODOR. Discovers *Bacterium coli*
1887 WEICHELBAUM, ANTON. Discovers *Meningococcus*
1888 ROUX, ÉMILE, and YERSIN, A. Discover diphtheria toxin
1890 BEHRING, EMIL VON, and KITASATO, SHIBAMIRO. Use antitoxin treatment for lockjaw and diphtheria
1894 ROUX, ÉMILE. Uses horses for production of antitoxin
1894 KITASATO, SHIBAMIRO, and YERSIN, A. Discover cause of bubonic plague
1894 PFEIFFER, RICHARD. Discovers bacteriolysis
1896 GRÜBER, MAX. Discovers bacterial agglutination
1896 WIDAL, FERNAND. Originates Widal test for typhoid
1896 WRIGHT, SIR ALMROTH. Makes typhoid vaccination practicable. Originates general vaccinothrapy
1897 ROSS, SIR RONALD. Proves that Anopheles mosquito transmits malaria
1897 SHIGA, ISAGIYOI. Discovers dysentery bacillus

- 1899 LÖFFLER, FREDERICK. Proves foot-and-mouth disease to be caused by filtrable virus, thus establishing this concept. Introduces preventive inoculation against this disease
- 1901 REED, W., CARROLL, J., AGRAMONTE, A., and LAZEAR, J. W. Make detailed study of yellow fever in Cuba. Produce disease experimentally. Carroll submits to mosquito inoculation. Lazear dies from accidental bite. Prove relationship of mosquito to yellow fever
- 1901 GORGAS, MAJOR WILLIAM. Frees Havana and Panama of yellow fever by screening patients and destroying mosquitoes. Great triumph in sanitation
- 1905 SCHAUDINN, FRITZ. Discovers *Treponema pallidum* as cause of syphilis
- 1906 BORDET, JULES, and GENGOU, OCTAVE. Discover bacillus of whooping cough
- 1906 RICKETTS, HOWARD T. Proves that tick transmits Rocky Mountain spotted fever. Demonstrates *Rickettsia* bodies (confirmed by Wolbach in 1916)
- 1907 WASSERMANN, AUGUST VON. Diagnoses syphilis by serum reaction
- 1913 BEHRING, EMIL VON. Discovers toxin-antitoxin treatment to prevent diphtheria
- 1913 SCHICK, BELA. Originates Schick test for diphtheria
- 1915 TWORT, F. W.; 1917 D'HERELLE, F. Independently discover bacteriophage
- 1917 DOCHEZ, A. R., and AVERY, J. T. Discover the polysaccharide nature of the bacterial capsule; important in immunology
- 1923 DICK, GEORGE and GLADYS. Prove *Streptococcus hæmolyticus* to be the cause of scarlet fever
- 1933 SMITH, M. M., ANDREWS, C. H., and LAIDLAW, P. P. Successfully transmit influenza to experimental laboratory animals

DEVELOPMENT OF AGRICULTURAL BACTERIOLOGY

- 1877 SCHLOESING, TH., and MÜNTZ, C. A. Show that nitrification is due to bacteria ("organized ferments")
- 1885 BERTHELOT, M. Establishes fact that nitrogen fixation is due to microorganisms in soil
- 1886 HELLRIEGEL, H., and WILFARTH, H. Discover symbiotic nitrogen-fixing bacteria on legumes
- 1888 BEIJERINCK, MARTINUS. Isolates *Rhizobium radicola*
- 1878 } WARINGTON, R. Shows that there are two stages in process of nitrification, and two organisms causing changes. Does not isolate them
- 1884 }
- 1891 }
- 1890 WINOGRADSKY, S. Is the first to obtain nitrifying organisms in pure culture. Names them *Nitrosococcus* and *Nitrosomonas*

APPENDIX B

STANDARD METHODS FOR THE EXAMINATION OF WATER¹

MATERIALS

A. WATER

Distilled water shall be used in the preparation of all culture media and reagents.

B. MEAT EXTRACT

Bacto-beef extract, or any other brand giving equivalent results, shall be used. Meat infusion shall not be used.

C. PEPTONE

Bacto-peptone, or any other peptone which comparative tests have shown to give equivalent results, may be used.

D. SUGARS

All sugars used shall be of the highest purity.

E. AGAR

The agar used shall be of the best quality. If not especially prepared for bacteriological work, agar should be soaked in distilled water and drained before use.

F. GELATIN

The gelatin used shall be of light color, shall not contain objectionable impurities, and shall be free from preservatives. The melting point shall be such that a 10 per cent standard nutrient gelatin shall melt at 25° C. or over.

G. GENERAL CHEMICALS

Special effort shall be made to have all the ingredients used for culture media chemically pure.

¹From "Standard Methods for the Examination of Water and Sewage" (eighth edition). American Public Health Association, 1936.

H. DYES

Dyes certified by the Commission on Standardization of Biological Stains for use in the preparation of media shall be employed.

PREPARATION OF CULTURE MEDIA

A. ADJUSTMENT OF REACTION

The reaction of culture media shall be stated in terms of hydrogen ion concentration. Tests to control the adjustment to the required hydrogen ion concentration may be made either electrometrically or colorimetrically. Colorimetric methods are satisfactory for this purpose.

The following example illustrates a recommended procedure:

Add 5 ml. of distilled water to each of two clean test tubes, similar in size, shape, and color to the tubes used for the color standards—6 inch by $\frac{1}{2}$ inch tubes are recommended. Withdraw 10 ml. of the medium to be adjusted and add 5 ml. to each of these two tubes. To one of these add 0.5 ml. of a solution of an indicator which will adequately cover the desired pH range. The amount of indicator in the test solution must be the same as in the standard. This is usually 0.5 ml. Using a comparator block, superimpose the tube containing the diluted medium plus the indicator over a tube of distilled water and superimpose the tube of diluted medium without indicator over the color standard of the pH desired. Titrate the tube of diluted medium plus indicator with an accurate 1:10 dilution of an approximately normal sodium hydroxide solution until the color, viewed through the distilled water tube matches the color of the pH standard as observed through the diluted medium without the indicator. Calculate the amount of normal sodium hydroxide solution which must be added to the medium to reach this reaction. After the addition and thorough mixing, check the reaction. The required final reaction is given in the directions for preparing each medium. If specific reaction is not described, adjustment is not necessary.

The increase in the hydrogen ion concentration during sterilization will vary slightly with the individual sterilizer in use and the initial reaction required in order to obtain the correct final reaction will have to be determined. The decrease in the pH reading will usually be between 0.2 and 0.4.

B. STERILIZATION

All media, except as directed for the preparation of sugar broths, shall be sterilized in the autoclave at 15 lbs. (120° C.) for 15 minutes after the pressure has reached 15 lbs. All air must be forced out of the autoclave by allowing live steam to stream through it for a few minutes before the pressure is allowed to rise. As soon as possible

after sterilization the medium shall be removed from the autoclave and cooled rapidly. Rapid and immediate cooling of gelatin and lactose media is imperative.

Media shall be sterilized in small containers, and these must not be closely packed together. No part of the medium shall be more than 2.5 centimeters from the outside surface of the glass or from the surface of the medium.

C. CLARIFICATION

The extent to which a medium should be clarified depends upon the use to which the medium is put, and will vary also according to the experience and preference of the laboratory worker. Any method of clarification is allowable which will yield a medium sufficiently clear for the detection of bacterial growth and at the same time will not remove necessary nutritive ingredients. Methods may include clarification in a centrifuge or filtration through paper, cotton, cheese-cloth or towels.

D. NUTRIENT BROTH

Add 3 g. of beef extract and 5 g. of peptone to 1000 ml. of distilled water. Heat slowly on a water bath to 65° C., stirring until dissolved. Make up the lost weight with distilled water. Adjust the reaction so that the pH reading after sterilization will be between 6.4 and 7.0.

Bring to a boil over a free flame, cool to 25° C., make up the lost weight with distilled water and clarify. Distribute in test tubes, 10 ml. to each tube, or in other desired containers. Sterilize as directed under *B*.

E. LACTOSE BROTH

To nutrient broth as prepared in *D*, add 0.5 per cent of lactose.

Adjust the reaction so that the pH reading after sterilization will be between 6.4–7.0, preferably at 6.9. Place in fermentation tubes and sterilize as directed under *B*, provided that total time of exposure to any heat is not more than one-half hour. Cool rapidly after removal from autoclave.

If the above condition of exposure to heat cannot be fulfilled, prepare a 10 or 20 per cent solution of lactose in distilled water and sterilize as directed under *B*, or by heating in an Arnold sterilizer at 100° C. for 1½ hours. Add this solution to sterile nutrient broth in amount sufficient to make a 0.5 per cent lactose solution, tube with proper precautions for preserving its sterility, and sterilize at 100° C. for 30 minutes.

It is permissible to add by means of a sterile pipette directly to a tube of sterile nutrient broth enough of the lactose solution to make the required 0.5 per cent concentration. The tubes so made shall be incubated at 37° C. for 24 hours as a test for sterility before they are used.

F. NUTRIENT GELATIN

Add 3 g. of beef extract, 5 g. of peptone and 120 g. of gelatin (undried market product as stored in the ordinary laboratory cupboard) to 1000 ml. of distilled water.

Heat slowly on steam bath to 65° C. until all ingredients are dissolved.

Make up the lost weight with distilled water and adjust the reaction so that the pH reading after sterilization will be between 6.4 and 7.0.

Bring to a boil, stirring vigorously. Make up the lost weight with distilled water and clarify.

Distribute in the desired containers, and sterilize as directed under B.

G. NUTRIENT AGAR

Add 3 g. of beef extract, 5 g. of peptone and 15 g. of agar (undried market product as stored in the ordinary laboratory cupboard) to 1000 ml. of distilled water. Boil until all the agar is dissolved. Make up the lost weight with hot distilled water. Adjust the reaction so that the pH reading after sterilization will be between 6.4 and 7.0.

Bring to a boiling temperature, stirring frequently. Restore the lost weight with hot distilled water, and clarify.

Distribute in the desired containers, and sterilize as directed under B.

H. ENDO MEDIUM

1. *Preparation of stock agar.* Add 5 g. of beef extract, 10 g. of peptone and 30 g. of agar to 1000 ml. of distilled water. The undried market type of agar as stored in the ordinary laboratory cupboard may be used if desired.

Boil until the agar is dissolved and make up lost weight due to evaporation with distilled water.

Adjust the reaction so that the pH reading after sterilization will be 7.4.

Clarify if desired.

Add 10 g. of lactose and dissolve.

Place in small flasks or bottles 100 ml. to each, and sterilize in autoclave as directed under *B*.

2. *Preparation of plates.* Prepare a 3 per cent solution of certified basic fuchsin in 95 per cent ethyl alcohol.

Allow to stand 24 hours and filter.

Melt lactose agar as prepared above, and to each 100 ml. add 1 ml. of the 3 per cent basic fuchsin solution and 0.125 g. of anhydrous sodium sulfite dissolved in 5 ml. of distilled water. The sulfite solution must be freshly prepared.

Mix thoroughly, pour plates with usual precautions against contamination and allow to harden.

The medium should be light pink when hot and almost colorless when cool. As batches of fuchsin differ somewhat in dye content, it is possible that the medium made up according to this formula may be too highly colored before incubation or may not give the proper reaction when seeded with colon bacilli. In such a case, the strength of the basic fuchsin solution may be varied.

I. EOSIN METHYLENE BLUE AGAR

Add 10 g. of peptone, 2 g. of dipotassium phosphate (K_2HPO_4) and 15 g. of undried agar to 1000 ml. of distilled water.

Boil until all ingredients are dissolved and make up loss due to evaporation with distilled water.

Adjustment of reaction is not necessary.

Place measured quantities (100 or 200 ml.) in flasks or bottles and sterilize in the autoclave as directed under *B*.

To prepare plates, melt stock agar as described above and to each 100 ml. add 5 ml. of sterile 20 per cent aqueous lactose solution, 2 ml. of 2 per cent aqueous solution of eosin, yellowish, and 2 ml. of 0.05 per cent aqueous solution of methylene blue.

Mix thoroughly, pour into Petri dishes and allow to harden.

It is permissible to add all the ingredients to the stock agar at the time of preparation, place in tubes or flasks, and sterilize. Plates may be prepared from this stock. Discolorization of the medium occurs during sterilization. The color returns after cooling.

J. BRILLIANT GREEN LACTOSE BILE

Dissolve 10 g. of peptone and 10 g. of lactose in not more than 500 ml. of distilled water. Add 200 ml. of fresh ox bile or 20 g. of dehydrated ox bile dissolved in 200 ml. of distilled water. The solution of dehydrated ox bile shall have a pH between 7.0 and 7.5.

Make up with distilled water to approximately 975 ml.

Adjust the reaction to a pH reading of 7.4.

Add 13.3 ml. of a 0.1 per cent solution of brilliant green in distilled water.

Add sufficient distilled water to make the volume 1000 ml.

Filter through cotton.

Distribute in fermentation tubes and sterilize as directed under *E*.

The reaction after sterilization (determined by potentiometric and not by colorimetric methods) should be not less than pH 7.1 and not more than pH 7.4.

M. FORMATE RICINOLEATE BROTH

Add 5 g. of peptone, 5 g. of lactose, 5 g. of sodium formate and 1 g. of sodium ricinoleate to 1000 ml. of distilled water.

Heat slowly on a water bath with constant stirring until dissolved.

Add distilled water to make the volume 1000 ml.

Adjust the reaction so that the pH reading after sterilization will be 7.3-7.5.

Distribute in fermentation tubes and sterilize at 11 to 13 pounds for 15 minutes.

SAMPLES

A. COLLECTION

Samples for bacterial analysis shall be collected in bottles which have been cleansed with great care, rinsed in clean water, and sterilized.

Great care must be exercised to have the samples representative of the water to be tested and to see that no contamination occurs at the time of filling the bottles or prior to examination.

B. STORAGE AND TRANSPORTATION

Because of the rapid and often extensive changes which may take place in the bacterial flora of bottled samples when stored even at temperatures as low as 10° C., it is urged, as of importance, that all samples be examined as promptly as possible after collection.

The time allowed for storage or transportation of a bacterial sample between the filling of the sample bottle and the beginning of the analysis should not be more than six hours for impure waters and not more than twelve hours for relatively pure waters. During the period of storage, the temperature shall be kept between 6° C.

and 10° C. Any deviation from the above limits shall be so stated in making reports.

When finished water samples containing residual chlorine are to be stored or shipped before examination, it is important to use some method for destroying the chlorine. Because of the relatively small amount of accurate data available, it is not possible to recommend any particular method at the present time. Each set of conditions represents an individual problem.

This warning is particularly important in the transportation of samples from chlorinated swimming pools.

DILUTIONS

Dilution bottles or tubes shall be filled with the proper amount of water so that after sterilization they shall contain exactly 9 ml. or 99 ml. as desired. The exact amount of water can be determined only by experiment with the particular autoclave in use. If desired, the 9 ml. dilution may be measured out from a flask of sterile water with a sterile pipette.

The water used for dilution shall be tap water, or the phosphate dilution water recommended in the procedure for biochemical oxygen demand. Distilled water shall not be used.

Dilution bottles shall be sterilized in the autoclave at 15 lbs. (120° C.) for 15 minutes after the pressure reaches 15 lbs.

The sample bottle shall be shaken vigorously 25 times and 1 ml. withdrawn and added to the proper dilution bottle or tube as required. Each dilution bottle or tube after the addition of the 1 ml. of the sample shall be shaken vigorously 25 times before a second dilution is made from it, or before a sample is removed for plating.

PLATING

All sample and dilution bottles shall be shaken vigorously 25 times before samples are removed for plating. Plating shall be done immediately after the dilutions are made. One ml. of the sample or dilution shall be used for plating and shall be placed in the Petri dish first. Ten ml. of liquefied medium at a temperature of 42° C. shall be added to the 1 ml. of water in the Petri dish. The cover of the Petri dish shall be lifted just enough for the introduction of either the pipette or culture medium, and the lips of all test tubes or flasks used for pouring the medium shall be flamed. The medium and sample in the Petri dish shall be thoroughly mixed and uniformly spread over the bottom of the Petri dish by tilting and

rotating the dish. All plates shall be solidified as rapidly as possible after pouring and placed immediately in the appropriate incubator. Endo plates or eosin methylene blue plates shall be made by placing one loopful of the material to be tested on the surface of the plate and distributing the material uniformly with a sterile wire or glass rod.

INCUBATION

Gelatin plates shall be incubated for 48 hours at 20° C. in a dark well-ventilated incubator in an atmosphere practically saturated with moisture.

Agar plates may be used for counts made either at 20° C. or 37° C. The time for incubation at 20° C. shall be 48 hours and at 37° C. 24 hours. The incubator shall be dark, well ventilated and the atmosphere shall be practically saturated with moisture. Glass covered plates shall be inverted in the incubator. Any deviation from the above described method shall be stated in making reports. Plates shall not be closely packed.

In making report of the water examination the medium used for the total count should be stated, that is, whether gelatin or agar, and the temperature of incubation given.

COUNTING

In preparing plates, such amounts of the water under examination shall be planted as will give from thirty to three hundred colonies on a plate, and the aim should be always to have at least two plates giving colonies between these limits. Where it is possible to obtain plates showing density of colonies within these limits, only such plates should be considered in recording results, except where the same amount of water has been planted in two or more plates, of which one gives colonies within these limits, while others give less than thirty or more than three hundred. In such case, the result recorded should be the average of all the plates planted with this amount of water. Ordinarily, it is not desirable to plant more than 1 ml. of water in a plate; therefore, when the total number of colonies developing from 1 ml. is less than thirty, it is obviously necessary to record the results as observed, disregarding the general rule given above.

Counting shall be done with a lens giving a magnification of approximately $2\frac{1}{2}$ diameters. In order to avoid fictitious accuracy and yet to express the numerical results by a method consistent with the precision of the work, the number of colonies of bacteria per ml. shall be recorded. (See table, p. 73.)

TEST FOR THE PRESENCE OF MEMBERS OF THE
COLI-AEROGENES GROUP

A. INTRODUCTION AND DEFINITIONS

1. It is recommended that the coli-aerogenes group be considered as including all aerobic and facultative anaerobic Gram negative non-spore-forming bacilli which ferment lactose with gas formation. The coli-aerogenes group as defined above is equivalent to the "B. coli" group as used in all editions of Standard Methods of Water Analysis prior to the sixth edition, and to the colon group described in the current edition of Standard Methods of Milk Analysis.

The standard tests for the coli-aerogenes group shall be either the Presumptive, the Confirmed, or the Completed test as hereafter defined, each test being applicable under the circumstances specified in *E*, below.

2. Presumptive test. The formation of gas in a standard lactose broth fermentation tube at any time within 24 hours with incubation at 37° C. or the formation of gas during a second 24 hour period with confirmation as described in 3, is presumptive evidence of the presence of members of this group, since the majority of the bacilli which give such reaction belong to the group.

3. Confirmed test. The formation of gas at any time within 48 hours with incubation at 37° C. in a fermentation tube containing a specified liquid confirmatory medium which has been seeded from a lactose broth fermentation tube in which gas has formed, or the appearance of aerobic lactose splitting typical colonies on a specified solid confirmatory medium made from a lactose fermentation tube in which gas has formed, confirms the presumption that gas formation in the fermentation tube was due to the presence of members of the coli-aerogenes group.

4. Completed test. To complete the demonstration of the presence of organisms of this group, it is necessary to show that one or more aerobic plate colonies consist of Gram negative non-spore-forming bacilli, which, when inoculated into a lactose broth fermentation tube, form gas.

5. In reporting results, the particular test (presumptive, confirmed or completed) applied to the sample should be recorded.

6. When it is desired to differentiate between the coli and the aerogenes sections of the coli-aerogenes group, the detailed procedure shall not follow primary planting in liquid media, but shall be based upon primary planting of the sample in solid media.

B. PRESUMPTIVE TEST

1. Inoculate a series of broth lactose fermentation tubes with appropriate graduated quantities of the water to be tested. Each fermentation tube must contain at least twice as much medium as the portion of sample to be tested, or the concentration of peptone and lactose in the mixture of medium. The portions of the water sample used for inoculating the lactose broth fermentation tubes will necessarily vary in size with the character of the water under examination.

2. Incubate the fermentation tubes at 37° C. for 48 hours unless gas appears earlier. Examine each tube at the end of 24 hours and if no gas then appears, again at the end of 48 hours. Record presence or absence of gas formation at each examination of the tubes.

More detailed records of the amount of gas formed, though desirable for the purpose of study, are not necessary for carrying out the standard tests prescribed.

3. Formation within 24 hours of gas in the inverted vial in the fermentation tube constitutes a *positive presumptive test*.

4. If no gas or only a small bubble of gas is formed in 24 hours, the incubation shall be continued to 48 hours. If gas in any quantity is present after the second but not the first 24 hour incubation period, the test is considered as doubtful and the presence of organisms of the coli-aerogenes group should be confirmed. Confirmed tubes may then be given the same consideration in the presumptive test as those showing gas formation in the first 24 hour incubation period.

5. The absence of gas formation after 48 hours incubation constitutes a negative test. (An arbitrary limit of 48 hours observation doubtless excludes from consideration occasional members of the coli-aerogenes group which form gas very slowly, but for the purpose of a standard test the exclusion of these occasional slow gas-forming organisms is considered immaterial.)

C. CONFIRMED TEST

The use of Endo or eosin methylene blue plates or one of the following liquid confirmatory media is permitted: brilliant green lactose bile, crystal violet lactose broth, fuchsin lactose broth, formate ricinoleate broth. The order in which these media are named does not signify their relative value. For the purpose of this test, all are equivalent, but it is recommended that the laboratory worker base his selection of any one of the confirmatory liquid media upon correlation of the confirmed tests thus obtained with a series of completed tests.

1. *Endo or Eosin Methylene Blue Plates*

1.1: Streak one or more plates from a tube which shows gas formation in lactose broth from the smallest amount of water tested. It is desirable to make transfers as soon as possible after gas formation occurs. In order to obtain typical results it is essential that the plates be streaked so as to insure the presence of some discrete colonies. Transfers should be made not later than the end of the first 24 hour incubation period if gas has formed

during this time. If at the end of 48 hours gas has formed in tubes containing less of the sample of water than at 24 hours, transfers should be made from these tubes. (For example, if the water has been tested in amounts of 10 ml., 1 ml., and 0.1 ml., and gas is formed in 10 ml. and 1 ml., not in 0.1 ml., the test need be confirmed only in the 1 ml. amount. But if the larger amounts are not transplanted for confirmation, they shall be recorded as confirmed, even though the smaller portion may fail subsequently to confirm.)

1.2. Incubate the plates at 37° C. for 18 to 24 hours.

1.3. Results, typical and atypical. If typical colonies have developed upon the plate within this period, the confirmed test may be considered positive. If, however, no typical colonies have developed within 24 hours, the test cannot yet be considered definitely negative, since it not infrequently happens that members of the coli-aerogenes group fail to form typical colonies on Endo or eosin methylene blue plates, or that the colonies develop slowly. In such case, it is always necessary to complete the test as directed under *D*.

2. Liquid Confirmatory Media

2.1. Transfer from a lactose broth tube showing gas to the fermentation tube containing the selected confirmatory medium. It is recommended that all lactose broth tubes showing gas be confirmed to comply with this test, but it is allowable to follow the recommendations in *C*, 1. Transfers should be made as soon as gas appears within the 48 hour incubation period. In routine testing it is convenient to make observations and transfers if required at the end of 24 and 48 hours. In making transfers from the lactose broth fermentation tubes showing gas, the tube shall first be gently shaken or mixed by rotating and the transfer shall be made by means of a wire loop, the loop to be not less than 3 mm. in diameter; or it is permissible to use a sterile capillary tube or sterile pipette when it is considered desirable to transfer larger amounts.

2.2. Incubate the inoculated tubes containing the confirmatory medium for 48 hours at 37° C.

2.3. The formation and presence of gas in any amount in the inverted vials in the fermentation tubes at any time within 48 hours constitutes a confirmed test.

D. COMPLETED TEST

1. Procedure

1.1. Streak one or more Endo or eosin methylene blue plates from the lactose broth tubes which show gas from the smallest amount of water tested or from the secondary selective medium tubes which show gas. If these plates are streaked from the original lactose broth tube they should be made at the same time that the secondary selective medium tubes are planted. If plates are used for the confirmed test, completion may be continued after incubation.

1.2. Incubate the plates at 37° C. for 18 to 24 hours.

1.3. Identification. From the plates fish one or more typical colonies, or if no typical colonies are present, fish two or more colonies considered most likely to be organisms of the coli-aerogenes group, transferring each to an agar slant and a lactose broth fermentation tube. The lactose broth fermentation tubes thus inoculated shall be incubated until gas formation

is noted — the incubation not to exceed 48 hours. The agar slants shall be incubated at 37° C. for 24 hours, when a microscopic examination for the presence of spores and a Gram stain of at least one culture shall be made, selecting when possible the one which corresponds to one of the lactose broth fermentation tubes which has shown gas formation. If none of the lactose broth fermentation tubes contain gas at the end of 24 hours, all the agar slants shall be examined, and corresponding tubes examined on the following day.

1.4. Results. The formation of gas in lactose broth, and the demonstration of Gram negative non-spore-forming bacilli in the agar culture, shall be considered a satisfactory completed test, demonstrating the presence of a member of the coli-aerogenes group.

The absence of gas formation in lactose broth, or failure to demonstrate Gram negative non-spore-forming bacilli in a gas forming culture, constitutes a negative test.

When spore-forming lactose fermenting organisms are found, the culture should be further studied to ascertain the possible presence of bacteria of the coli-aerogenes group with the spore-bearing organisms. This may be done by transferring the culture to formate ricinoleate broth and incubating at 37°C. for 48 hours. If no gas is produced only spore forming lactose fermenters are present. If gas is produced in the formate ricinoleate broth, members of the coli-aerogenes group may be present. If gas is produced, the presence of coli-aerogenes group organisms should be verified by inoculation of a tube of standard lactose broth and an agar slant. If, after 48 hours, gas is produced in the former and no spores are present in the latter, the test may be deemed "completed." If spores are present, for practical purposes the organisms of the coli-aerogenes group may be considered absent.

E. SELECTION OF COLI-AEROGENES TESTS

1. The laboratory worker, when he elects to apply either the Presumptive, the Confirmed, or the Completed test for the coli-aerogenes group, shall be guided by the following basic considerations.

1.1. The Completed Test shall be applied to the smallest gas-forming portion or portions of:

- any sample of drinking water upon which sufficient data are not available to justify the application of the confirmed test;
- any sample of drinking water upon which the previous records indicate the inapplicability of the confirmed test;
- any sample of drinking water which is being examined with reference to the U. S. Treasury Standard.

1.2. The Confirmed Test may be applied to the smallest gas-forming portion or to all portions showing gas, from:

- any sample of raw water, water in process of purification, or water prepared for human consumption where a sufficient number of prior examinations have established a satisfactory correlation of the Confirmed test with the Completed test;
- miscellaneous samples from any other source when it is known that the presumptive test is too broadly inclusive.

1. 3. The Presumptive Test may be applied to the smallest gas-forming portion or portions:

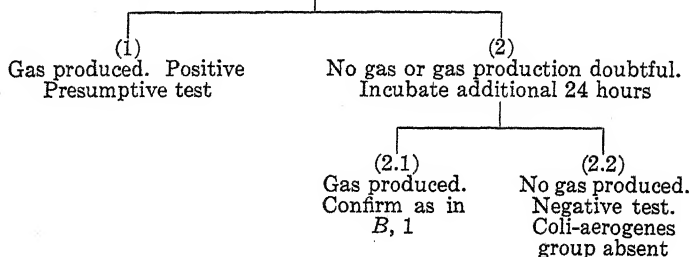
in the examination of sewage, sewage effluents, or water showing relatively high pollution, where fitness for use as drinking water is not under consideration, and

in the routine examination of raw waters in purification plants, provided that records indicate that the Presumptive Test is not too inclusive for the production of data statistically comparable to that obtained upon the finished water.

SCHEMATIC OUTLINE OF PRESUMPTIVE, CONFIRMED AND COMPLETED TESTS

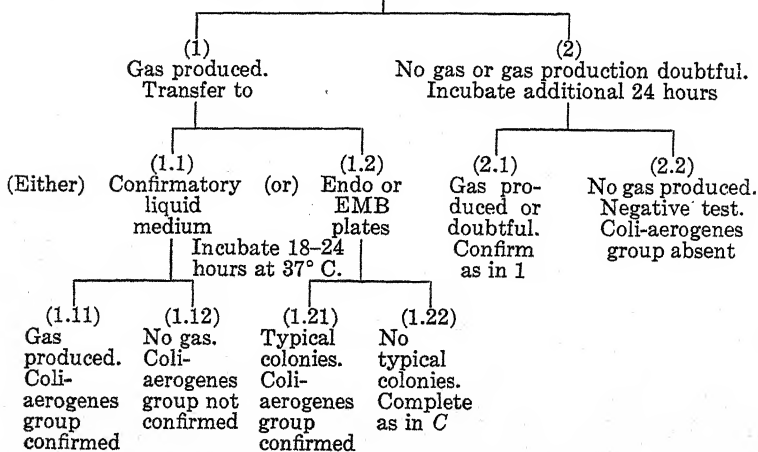
A. PRESUMPTIVE TEST

Inoculate lactose broth fermentation tubes and incubate 24 hours at 37° C.



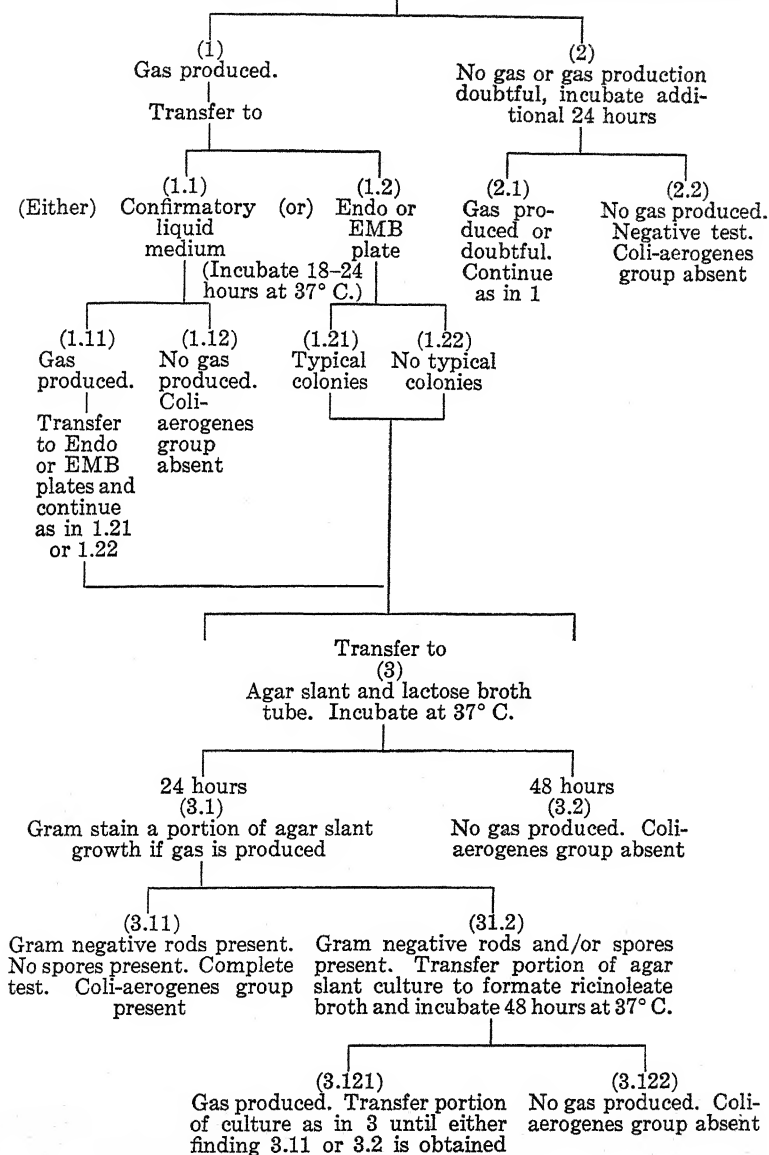
B. CONFIRMED TEST

Inoculate lactose broth fermentation tubes and incubate 24 hours at 37° C.



C. COMPLETED TEST

Inoculate lactose broth fermentation tubes and incubate 24 hours at 37° C.



ESTIMATION OF COLI-AEROGENES GROUP DENSITY

1. Tests for the presence of and density of coli-aerogenes group organisms shall be based upon the primary inoculation into lactose broth of one or more portions of one or more decimal dilutions of the sample.

The number of portions planted and the range of dilutions made will depend upon the presumed character of the water under examination, as well as the records or absence of records of previous samples. Every fermentation tube shall contain such an amount of medium and such an initial concentration of ingredients, that after the addition of the sample, not more than 0.5 per cent nor less than 0.3 per cent of peptone and/or lactose are present in the mixture.

2. When examining water of presumed drinking water quality, inoculate a series of lactose broth fermentation tubes with appropriate graduated quantities of the water to be tested. The amounts of sample selected for inoculating should be such that the largest portions will result in gas production in all or the majority of broth tubes into which they are inoculated, and such that the smallest portions will result in no gas production in all or the majority of broth tubes into which these smallest portions are inoculated. Because the numerical value of the bacterial content is largely determined by the analytical result of that dilution or dilutions of the sample intermediate between the above mentioned ones, the greater number of tube plantings should be made of this intermediate or critical portion. The number of such critical portions to be inoculated will be governed by the desired accuracy of the result (and in general should be not less than 5), while the numbers of the largest and smallest portions to be inoculated will be governed by the probable accuracy of the estimate of the bacterial content at the time the series of dilutions is decided upon. Inasmuch as definite indications of the occurrence of occasional higher densities of the coli-aerogenes group are desirable and are facilitated by multiple plantings of the highest dilution, more than one and up to five such portions may be inoculated. Practical considerations limit the size of the largest portion to 100 ml. and when 100 ml. portions are inoculated, it is possible that gas production will not result in the majority of broth tubes.

3. When examining water for evidence of conformance to the U. S. Treasury Drinking Water Standard, inoculate a series of 5 lactose broth fermentation tubes, each with a 10 ml. portion of

the sample. The complete test shall be made upon these samples. The results may be recorded either in terms of the number of positive portions of each sample examined or in terms of the most probable number of coli-aerogenes group organisms present in the sample examined.

4. When examining water of other than drinking water quality, inoculate a series of lactose broth fermentation tubes with appropriate graduated quantities of the water to be tested. The principles stated in 2 relating to the selection of portions to be examined, apply here. The selection of the size of the portions tested will depend upon the probable degree of coli-aerogenes group density in the sample and will be governed by the experience of the technician with the character of the water under examination.

5. The number of positive findings of coli-aerogenes group organisms (either confirmed or completed) resulting from multiple portion decimal dilution plantings made according to procedure 2 or 3 above, shall be computed and recorded in terms of the "Most Probable Number" (M.P.N.). The most probable number results for a variety of planting series and positive results are shown in Table 18.

The quantities indicated at the head of the columns relate more specifically to finished waters. The figures may be used in computing M.P.N. in larger or smaller portion plantings in the following manner: If instead of 10, 1.0 and 0.1 ml. portions, a combination of 100, 10 and 1 ml. portions is used, the M.P.N. may be recorded as 1/10 the figure in the table. If, on the other hand, a combination of corresponding portions of 1.0, 0.1 and 0.01 ml. is planted, record 10 times the figure in the table; if a combination of 0.1, 0.01 and 0.001 ml. portions is planted, record 100 times the figure in the table.

The most desirable procedure for obtaining a single numerical value for a series of analytical results is to express the results of each analysis in terms of its M.P.N. value and strike an arithmetic average of these values. It is mathematically incorrect to summarize the number of positive tubes in the various dilutions for a number of samples from the same source on the same day, or over a period of days, and use an arithmetic average of the accumulated figures to determine a single M.P.N. for the series of samples.

6. While it is recognized that the planting of single portions of several dilutions of a water sample affords less adequate information as to coli-aerogenes density than is desirable, such procedure is recognized as a minimum, if other conditions make such procedure

TABLE 18. MOST PROBABLE NUMBERS—COLI-AEROGENES TESTS

[Most probable numbers per 100 cc. of sample, planting 5 portions in each of 3 dilutions in geometric series.]

(1)			(2)	(1)			(2)	(1)			(2)	(1)			(2)	(1)			(2)	(1)			(2)	(1)			(2)	(1)			(2)
10	1	0.1		10	1	0.1		10	1	0.1		10	1	0.1		10	1	0.1		10	1	0.1		10	1	0.1		10	1	0.1	
0	0	0		1	0	0	2	2	0	0	4.5	3	0	0	7.8	4	0	0	13	5	0	0	23	0	0	0	23	0	0	0	23
0	0	1	1.8	1	0	1	4	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31	0	1	1	31	0	1	1	31
0	0	2	3.6	1	0	2	6	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43	0	2	2	43	0	2	2	43
0	0	3	5.4	1	0	3	8	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58	0	3	3	58	0	3	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76	0	4	4	76	0	4	4	76
0	0	5	9	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95	0	5	5	95	0	5	5	95
0	1	0	1.8	1	1	0	4	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33	0	1	0	33	0	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46	0	1	1	46	0	1	1	46
0	1	2	5.4	1	1	2	8.1	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	84	0	2	2	84	0	2	2	84
0	1	3	7.2	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	110	0	3	3	110	0	3	3	110
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	130	0	4	4	130	0	4	4	130
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130	0	5	5	130	0	5	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49	0	2	0	49	0	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70	0	2	1	70	0	2	1	70
0	2	2	7.4	1	2	2	10	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	95	0	2	2	95	0	2	2	95
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120	0	2	3	120	0	2	3	120
0	2	4	11	1	2	4	15	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150	0	2	4	150	0	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180	0	2	5	180	0	2	5	180
0	3	0	5.6	1	3	0	8.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79	0	3	0	79	0	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110	0	3	1	110	0	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140	0	3	2	140	0	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180	0	3	3	180	0	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210	0	3	4	210	0	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250	0	3	5	250	0	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130	0	4	0	130	0	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	170	0	4	1	170	0	4	1	170
0	4	2	11	1	4	2	15	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220	0	4	2	220	0	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280	0	4	3	280	0	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350	0	4	4	350	0	4	4	350
0	4	5	17	1	4	5	22	2	4	5	28	3	4	5	40	4	4	5	69	5	4	5	430	0	4	5	430	0	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	240	0	5	0	240	0	5	0	240
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350	0	5	1	350	0	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540	0	5	2	540	0	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920	0	5	3	920	0	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1600	0	5	4	1600	0	5	4	1600
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81	5	5	5	—	0	5	5	—	0	5	5	—

(1) Number of positive tubes in each of these three dilutions. (2) M.P.N. per 100 ml.

necessary. When the single portion decimal dilution method of approximating coli-aerogenes group density is used, the reciprocal of the confirmed or completed findings shall be recorded as the "indicated number" (I.N.). In assembling such results, if a negative finding occurs in an amount larger than the smallest portion giving a positive result, record as positive the portion next larger than the smallest one giving positive finding. The following table illustrates the method of recording and averaging results of such tests.

In order that results as reported may be checked and carefully evaluated, it is necessary that the report should show not only the

RESULTS OF TESTS IN AMOUNTS DESIGNATED				INDICATED NUMBER OF ORGANISMS OF THE COLI-AEROGENES GROUP	
10 ml.	1 ml.	0.1 ml.	.01 ml.	per ml.	per 100 ml.
+	-	-	-	0.1	10
+	+	-	-	1.0	100
+	+	+	-	10.0	1,000
+	+	+	+	100.0	10,000
+	+	-	+	10.0	1,000
Totals (for estimating averages)				121.1	12,110
Average of 5 tests (adjusted to significant figures)				24.0	2,400

average number of organisms per ml., but also the number of samples examined; and, for each dilution, the total number of tests made, and the number (or per cent) positive.

INTERPRETATION OF COLI-AEROGENES GROUP RESULTS

It is not within the province of this text to suggest the proper interpretation of results obtained by the use of the methods herein specified as standard. Reference may be made to the U. S. Treasury Standard for drinking water on interstate carriers for a discussion of allowable density of organisms of the coli-aerogenes group.

The definition of the coli-aerogenes group as given includes organisms of both the so-called fecal and non-fecal types. At the present time, any attempt to evaluate a drinking water on the basis of a distinction between these two types is regarded as unwarranted.

The inclusion of these methods should not be construed as detracting from the value of the group tests as above described for the routine examination of water supplies.

APPENDIX C

STANDARD METHODS OF MILK ANALYSIS¹

COLLECTION OF SAMPLES FOR BACTERIOLOGICAL EXAMINATION

All collecting apparatus, glassware, pipettes, collecting tubes, bottles, etc. shall be sterilized at a temperature of not less than 160° C. for not less than one hour.

Each sample shall consist of at least 10 cc. of milk. Before taking the sample the milk shall be mixed as thoroughly as possible. If the original container can be inverted, the mixing of the milk should be done by inverting it several times. If this is impossible, the milk should be stirred with some sterile stirrer. Any stirrer already in the container may be used. If there is none in the container, the sampling pipette (or any other sterile article) may be used; but it shall be used for one container only until it is again sterilized.

A sample merely poured from a large can is not a fair sample unless the milk in the can is thoroughly stirred. Neither is a sample of mixed milk, taken after it is poured into an unsterilized weighing vat, a fair sample from which to judge the quality of the milk before it was poured into the vat. Samples shall be taken from cans by means of a glass or aluminum tube with straight sides, long enough to reach the bottom of the original container, and inserted, not too rapidly, with the top of the tube left open. The finger then placed on the top of the tube will make it possible to withdraw the tube full of milk and transfer it to the sampling bottle. The sampling bottle should be large enough to hold the entire contents of the tube, all of which must be reserved as the sample. Each tube shall be used for collecting a single sample only, and must be washed and sterilized before it is used again. If the sample is taken from a bottle, the bottle should be first shaken, to insure thorough mixing, and the milk may be poured into the sample bottle, although it is better here also to use a sampling tube.

¹ From "Standard Methods of Milk Analysis, Bacteriological and Chemical" (fifth edition). American Public Health Association, 1927.

If the temperature of the milk is desired, it should be taken from a different container from that used for the bacteriological sample, or after the bacteriological sample has been withdrawn. All records shall be made immediately after taking the sample. The milk sample shall be placed in a properly labeled bottle. The most convenient kinds of sample bottles are glass-stoppered or else closed with a cork-lined screw cap. Cotton plugs are not a satisfactory method of closure. The sample bottles shall be placed at once in cracked ice, so that the milk is promptly cooled to near the freezing point.

The samples shall be transferred to the laboratory as quickly as possible and shall be plated with as little delay as possible. The samples placed in cracked ice and water may be kept for several hours (twelve) without an appreciable increase in bacteria. If the plates are not made within four hours from the time of collection, the number of hours that have elapsed should be stated in the report. If the milk is kept at 4° C., a slight and somewhat variable increase may be found in from twelve to twenty hours. Up to twenty hours this will not be more than 20 per cent in normal cases. The larger increases may be expected in milk which has been stored at low temperatures for some time previous to sampling. Continued shaking of the milk during its transit to the laboratory tends to break the clumps into smaller masses and so increases slightly the number of colonies.

In the case of samples to be used for direct microscopic examination, icing may be dispensed with, under some conditions, where it is possible to add preservatives (formalin, two to three drops of a 40 per cent solution of formaldehyde for each 10 cc. of milk) to the samples as taken. Samples containing preservatives, that have been allowed to stand until the cream is compact, are not satisfactory, and are likely to give a lower count than fresh samples. Useful information regarding the bacterial quality of a given milk supply can, however, be obtained by microscopic examination of the composite samples to which preservatives have been added for testing for butter fat.

MACROSCOPIC COLONY COUNT (PETRI PLATE METHOD)

COMPOSITION OF MEDIUM

Standard beef extract agar shall be used for all routine work and shall contain the following ingredients. (See Appendix B, p. 286.)

Agar	1.5 %
(market, not oven dried)	
Beef extract	0.3 %
Peptone	0.5 %
Distilled water	

The reaction of the medium is to be between pH 6.2 and pH 7.0. If necessary to adjust the reaction, special attention is to be given to the H ion concentration, use being made of one of the indicators, brom thymol blue or brom cresol purple. (For adjustment of reaction see Appendix B, p. 287.)

PLATING

For miscellaneous milk samples, the character of which is not known, three dilutions shall be made; namely, 1:100, 1:1000, and 1:10,000. Where the character of the milk is known, the number of dilutions may be reduced. If the milk is pasteurized, certified, or known to be fresh and of high grade, the 1000 and 10,000 dilutions may be omitted and 1:10 dilutions may be prepared. If the milk is known to be old and of high bacterial count, the 100 and 1000 dilutions may be omitted and dilutions in excess of 10,000 prepared. In no case shall less than two plates be made from each sample. Where two satisfactory plates are obtained, it is advisable to count both of them.

The water used for dilutions may be placed in dilution bottles (99 cc., 49.5 cc., and 9 cc. are convenient sizes) and sterilized for one hour in an autoclave at a pressure of fifteen pounds. The bottles should be marked so that it can be determined that they have neither gained nor lost water during or subsequent to sterilization. Or the water may be sterilized in bulk, if kept in a properly guarded container, and subsequently measured directly into the dilution bottles with sterilized pipettes.¹

The dilution bottles should have glass or cork stoppers, or some other type of closing that makes shaking possible. Cotton plugs are a less satisfactory method of closing, because a small portion of the dilution water will soak into the cotton.¹

¹ From "Standard Methods of Milk Analysis" (fourth edition). 1923.

Straight-sided pipettes graduated to deliver 1 cc. are best. They may be either the two-mark or the one-mark style. In either case the errors of measurement are caused more by faulty calibration or by faulty manipulation of the pipette than by the particular form of pipette used. In using two-mark pipettes great care must be taken to see that the quantities used are exactly 1 cc., while many one-mark pipettes in use are calibrated to *contain* 1 cc. rather than to *deliver* 1 cc. Breakage of tips of the latter type of pipette also causes errors.¹

In making dilutions the original sample and each dilution bottle shall be rapidly shaken twenty-five times, each shake being an up-and-down excursion of about one foot (the entire shaking not to take longer than about seven seconds). After the final dilution, fill a pipette to the mark and allow the contents to run into an empty Petri dish, the end of the pipette touching the dish as the liquid runs out. If the pipettes are of the one-mark style, be sure that they are so manipulated as to deliver a full cubic centimeter. Use care to raise the cover only as far as necessary to insert the end of the pipette.

Pipettes should be placed immediately in water after using, to make subsequent cleaning easier.

The flasks (or test tubes) of agar shall be melted in boiling water or steam, and after melting shall be cooled to a temperature of between 40° and 45° C. before using.

Pour about 10 cc. of the melted agar into the diluted milk in each inoculated Petri dish, and by a gentle rotary motion thoroughly mix the agar and the diluted milk. As nearly as possible the same amount of agar should be poured into each Petri dish, so that the depth of agar will be uniform in all. If desired, 10 cc. may be measured out from the flask with a sterile pipette.

It is important that the plating shall be completed as rapidly as possible. The work should be so planned that no more than fifteen minutes shall elapse after the dilution of the milk and before the agar is poured into the Petri dishes; and in no case shall the interval be allowed to exceed twenty minutes.

In those cases where room temperatures are so warm that difficulty is experienced in getting a 1.5 per cent agar to harden satisfactorily before inverting the plates, facilities should be provided for cooling the plates. After the agar has been thoroughly hardened, place the Petri dishes in an incubator. The danger of spreaders may be reduced either by the use of clay tops or by inverting the plates.

¹ From "Standard Methods of Milk Analysis" (fourth edition). 1923.

INCUBATION

Only one period of incubation, and one temperature is regarded as standard, namely, forty-eight hours at 37° C. Piles of plates should not be packed too closely together, and in crowded incubators ventilation should be provided. Sufficient moisture should be provided to permit plates with the normal amount of agar in them to remain in the incubator for at least four days before the agar dries up.

COUNTING PLATES

If among the different dilutions there are plates containing from thirty to three hundred colonies, these should be counted, and the number, multiplied by the dilution, should be reported as the final count. All colonies on such plates should be counted, and the numbers from the different plates averaged. If there are no plates within these limits, the one that comes the nearest to three hundred is to be counted. No plate that contains less than twenty colonies shall be counted, unless it happens that there are no other plates. If the number of colonies on the plates to be counted is in excess of three hundred per plate, a part of the plate may be counted and the total number estimated; but such plates are admittedly overcrowded, and the counts are less than they should be.

Counting shall be done with a lens, and all recognizable colonies included. A lens magnifying $2\frac{1}{2}$ diameters (or what the opticians call a $3\frac{1}{2}$ X lens) is recommended for general use. In case any particles visible by this method are of doubtful nature, they should be examined with a compound microscope, to determine whether they are colonies or dirt specks.

COMMON SOURCES OF ERROR IN COUNTS

Agar plate "counts" per cubic centimeter are to be regarded as estimates of numbers rather than as exact counts, since only a portion of a cubic centimeter is used in preparing the plates. As such they are (like all estimates) subject to certain well-known and recognized errors whose size can be largely controlled by the care taken in the analysis. Among these errors are:

a. Failure of some of the bacteria to grow because the incubation temperature, or the composition or reaction of the medium, is not suitable.

b. Inaccuracies in measurement of the quantities used.

c. Mistakes in counting, recording data, computing results, and the like.

d. Incomplete sterilization or contamination of the plates, dilution waters, etc.

The possible errors caused by these things make it highly important for all routine laboratories to follow carefully a standard procedure.

Recent investigations make it clear that these largely controllable errors are not so likely to cause serious misconceptions of the accuracy of the results as are the errors due to the fact that bacteria in milk usually cling together in groups of from two to many hundreds of individuals. These groups are only partially broken apart by the shaking given in preparing the dilutions, so that at best the counts from the agar plates represent the number of isolated individuals and groups of two or more bacteria that exist in the final dilution water. Thus the colony counts from the plates are always much smaller than the total number of bacteria present. This error would not be troublesome if the groups were of constant average size; but the best information available shows that the groups in ordinary market milk commonly vary in size, so that they contain an average of from two to six individual bacteria, while other samples, such as those bearing long-chain streptococci, may show groups containing an average of twenty-five or even more individual bacteria. The irregularity of this error (whose size is not indicated in any way by the appearance of the plates) should be kept in mind in interpreting the results obtained.

REPORTS

Because of the fact that agar plate counts only represent a fraction of the total number of bacteria present, they should not be reported as showing the "number of bacteria per cubic centimeter." Accurately speaking, the counts from agar plates give the estimated number of colonies that would have developed on standard agar per cubic centimeter of milk if an entire cubic centimeter of milk had been used for inoculation. Because this statement of fact is cumbersome, and also because a certain ratio exists in each case between the colony count and the total number of bacteria, it has become a common practice to speak of the plate counts as showing the number of bacteria per cubic centimeter. This is very confusing now that microscopic methods of counting have been developed which permit counts of the actual bacteria to be made. The most frequent ratio between agar plate counts made by the standard plating

method and the total number of bacteria present has been found to be approximately 1:4.

It is therefore recommended that all agar plate counts obtained by the standard technique shall not be reported in the form "2,000,000 bacteria per cubic centimeter," but rather as follows: "standard plate count, 2,000,000." The latter form of expression shall be considered an abbreviated method of saying: "an estimated count of 2,000,000 colonies per cubic centimeter as obtained by standard methods." Moreover, analysts shall be careful to avoid giving a fictitious idea of the accuracy of the standard plate count. There is ample justification for thinking it sufficiently accurate to justify drawing conclusions as to the general quality of a given sample of milk; and when a series of samples from the same source are examined, the average result may permit much more specific conclusions to be drawn with confidence.

Specific data showing the actual percentage error in these counts have been difficult to obtain, and have only recently been obtained by means of comparisons made between microscopic and agar plate counts. The conclusions reached by Breed and Stocking are that the margin between two plate counts made from similar samples of market milk must be as great as one to five before it becomes a practical certainty that the larger count actually represents the larger number of bacteria.

It is, however, self-evident that, between any two samples, the one having the higher count probably contains the greater number of bacteria; and this probability can be made a practical certainty by the examination of a series of samples. It is therefore required that a series of samples, preferably four or more, be examined before judgment shall be rendered as to the general quality of a given milk supply. Under no conditions is the practice sanctioned of publishing exact counts from individual samples as showing the quality of a given milk supply.

All laboratories conforming to standard procedure will keep a record of the exact number of colonies developed on the plates that are counted, but will render their reports in round numbers only. Never use more than two significant left-hand digits in any report, raising the number to the next highest round number in any case, but never lowering it. Those wishing to be still more conservative may use a form of report such as "standard plate count less than 10,000," "standard plate count between 10,000 and 30,000," and the like.

MICROSCOPIC COUNT OF BACTERIA (BREED METHOD)

Various methods for counting bacteria in milk by microscopic examination have been described, but the method that is commonly described as a *direct microscopic examination* of a dried film of milk has been found to be the simplest and most reliable method of counting the bacteria as they exist in the milk itself. It is recognized in this report as a standard technique, of equal standing with the colony count from agar plates, where used for judging the quality of unpasteurized milk.

APPARATUS REQUIRED

In addition to a microscope, microscopic slides, stains, etc., the only special apparatus required is a capillary pipette which discharges 0.01 cc. of milk. The most satisfactory form of pipette is made from a straight piece of thick-walled capillary tubing with a bore of such a size that the single graduation mark is from $1\frac{1}{2}$ to $2\frac{1}{2}$ inches from the tip. The tip shall be blunt, and of such a form that it will discharge the milk cleanly, without running back on the side of the tip. Pipettes of this type are now listed by all of the usual supply houses. The pipettes shall be calibrated so as to *deliver* 0.01 cc., not to *contain* 0.01 cc. The calibration of all pipettes shall be tested by weighing the amount of milk discharged on chemical balances. The weight for milk should be 0.0103 gram.

Only a single pipette is needed in making a series of tests, provided this is kept clean while in use. In this kind of work, cleanliness of glassware is more important than sterilization. Clean towels may be used for wiping the exterior of the pipettes while making the microscopic preparations, and their bores may be kept clean by rinsing them in clean water between each sample. The small amount of water left in the bore may be rinsed out in the milk sample under examination. This method of procedure, while adding a small number of bacteria to each sample, introduces only a theoretical error, tests showing that such bacteria cannot subsequently be detected, and make no difference in the final result. After use, the pipettes should be kept in a glass-cleaning solution, such as the commonly used mixture of sulphuric acid and potassium bichromate.

Routine laboratories will find it convenient to use larger microscopic slides than the ordinary 1-by-3-inch slide. The largest slides that have been found to be conveniently examined with the use of

a mechanical stage are cut 2 by $4\frac{1}{2}$ inches. Such slides may be stored in ordinary card-catalogue cases, and may be very cheaply prepared from thin window glass or old photographic negatives. A margin of ground or etched glass on the longer edges of the slide, about $\frac{1}{4}$ inch in width, allows lead-pencil labeling. The margins may be ground with an emery wheel or they may be etched with hydrofluoric acid. Special cardboard guide plates (size, 2 by $4\frac{1}{2}$ inches), marked off with 16-square-centimeter areas, are also needed. These are used as guide plates underneath the slides on which the milk preparations are made.

PREPARATION OF FILMS OF DRIED MILK

After a thorough shaking of the sample, 0.01 cc. of milk or cream shall be deposited upon a clean glass slide by means of the pipette above described. Spread the drop of milk uniformly over an area of one square centimeter by means of a clean, stiff needle. This may be most conveniently done by placing the slide upon the guide plate just described, or upon any other form of guide plate of glass or paper which is ruled in square-centimeter areas. The marks showing through the glass serve as guides. After spreading, the preparation shall be dried in a warm place, upon a level surface protected from dust. In order to prevent noticeable growth, this drying must be accomplished within five to ten minutes; but excessive heat must be avoided, or the dry films may crack and peel from the slide in later handling.

After drying, the slides are to be dipped in xylol — or any other suitable fat solvent — for a sufficient time to remove the fat (at least one minute), then drained, and again dried. After this, the slides are to be immersed in 90 per cent grain or denatured alcohol for one or more minutes, and then transferred to a solution of Löffler's methylene blue prepared as follows: Saturated alcoholic solution of methylene blue, 30 cc.; caustic potash in a 0.01 per cent solution, 100 cc.

Some of the methylene blue sold since the war has been found to be unsatisfactory in that solutions dissolve the milk films, or wash them with an even blue color in which the bacteria fail to show distinctly. This difficulty has now been corrected in all methylene blue sold under the certificate of the Commission on Standardization of Biological Stains. Old or unfiltered stains are to be avoided, as they may contain troublesome precipitates.

The slides are to be left in the stain until overstained. They are then to be rinsed in water and decolorized in alcohol. The decolorization takes from several seconds to a minute or more, during which time the slide should be under observation in order that the decolorization may not proceed too far. When properly decolorized, the background of the film should show a faint blue tint. Poorly stained slides may be decolorized and restained without injury. After drying, the slides may be examined at once, or they may be preserved indefinitely.

STANDARDIZATION OF THE MICROSCOPE

The microscope used must be so adjusted that each field covers a certain known fraction of the area of a square centimeter. The adjustment is simple if a micrometer slide, ruled in hundredths of a millimeter, is at hand (it is sometimes called a stage micrometer, as it is used under the objective on the stage of the microscope). The microscope should have a 1.9-millimeter ($\frac{1}{12}$ -inch) oil-immersion lens, and an ocular giving approximately the field desired (for example, a 6.4 X ocular). It should also be fitted with a mechanical stage. If the large slides described above are used, this must be a special stage, allowing a larger area of the slide to be examined than can be viewed with the usual mechanical stage.

To standardize the microscope, place the stage micrometer on the stage of the microscope, and by selection of oculars or by adjustment of the draw tube, or both, bring the diameter of the whole microscopic field to 0.205 mm. When so adjusted, each field of the microscope covers an area of approximately $\frac{1}{30000}$ cm. (actually $\frac{1}{30288}$ cm.). This means that the dried milk solids from $\frac{1}{30000}$ part of a cubic centimeter of milk are visible in each field of the microscope. Therefore, if the bacteria in one field only are counted, the number found should be multiplied by 300,000 to give the estimated number of bacteria per cubic centimeter. In practice, however, more than a single field is examined, so that the number used for multiplication is smaller than this.

As the microscopic examinations must be made with greater care where the bacteria are relatively few in number, it is required that in grading low-count milk a special ocular micrometer, with a circular ruling divided into quadrants, shall be used. In using this micrometer the microscope shall be so adjusted that the diameter of the circle on the eyepiece micrometer shall be 0.146 mm. In this case the amount of dried milk solids examined in each field of

the microscope is $\frac{1}{60000}$ part of a cubic centimeter of milk. The limitation of the examination of the slide to the central portion of each field avoids using the margins of the field where definition is hazy, and lessens the danger of overlooking bacteria. Likewise, the magnification used is greater than that used where the whole field is examined.

COUNTING AND GRADING MILK

The number of fields of the microscope to be examined varies with the character of the milk, and with the character of the data desired. Experience has shown that where the purpose is primarily to detect and eliminate the worst milk from ordinary market-milk supplies, it is entirely permissible to use the entire field of the microscope for examination. At least thirty representative fields of the microscope should be examined for each sample of milk. Where the average number of individual bacteria (not groups of bacteria) is less than 24 bacteria per 30 fields ($\frac{4}{5}$ of a bacterium per field), it may be assumed that the milk will ordinarily give a standard plate count of less than 60,000 per cubic centimeter. Where the number is less than 80 in 30 fields (an average of less than $2\frac{2}{3}$ bacteria per field), it may be assumed that the standard plate count will be less than 200,000 per cubic centimeter. Where the number is less than 800 per 30 fields (an average of less than $26\frac{2}{3}$ per field), it may be assumed that the standard plate count will not exceed one to two million per cubic centimeter.

Where counts are made in order to enforce more stringent standards, as at Grade A plants or as a basis for premiums on milk giving a standard plate count of less than 10,000 per cubic centimeter, the special eyepiece micrometer described above shall be used, and the microscope so adjusted that only the central portion of ~~each~~ field is examined for counting. Where less than 4 bacteria are found in 60 fields (an average of less than $\frac{1}{15}$ of a bacterium per field), it may be assumed that the milk would ordinarily give an official plate count of less than 10,000 per cubic centimeter. The grading of milk of this type must be done with especial care, as persons inexperienced with microscopic work have been found readily to confuse extraneous objects with bacteria in milk containing very few organisms. Where the number is less than 24 per 60 fields (an average of less than $\frac{2}{5}$ of a bacterium per field), it may be assumed that the standard plate count will be less than 60,000 per cubic centimeter. Where the number is less than 80 per 60 fields (an average of less

than $1\frac{1}{3}$ bacteria per field), it may be assumed that the standard plate count will be less than 200,000 per cubic centimeter. Where the number is less than 800 per 60 fields (an average of less than $13\frac{1}{3}$ bacteria per field), it may be assumed that the standard plate count will be less than one to two million.

The standards given are computed (with the exception of the poorest grades) on the assumption that the standard plate count will normally *average* one fourth of the total number of individual bacteria present. As many cases will be found which diverge markedly from the *average*, it is self-evident that this average represents only an approximation to the real conditions in any specific case, so that in some cases the microscopic grading will be more severe than that based on the plate counts, and *vice versa*. There is still a lack of sufficient data from which to judge fairly which system of grading is the more accurate. The indications are, however, that where the work is done with equal skill and care, and the allowances indicated are made, a reasonably close agreement in grade will be secured. This fact is highly reassuring as to the general accuracy of both systems of grading.

—In the routine grading of milk by the microscopic method it is not expected that exact counts will be made. A high-grade milk will show field after field of the microscope in which no bacteria are seen, while a poor grade of milk will show numerous bacteria in every field examined. It is only where the number of bacteria present is close to the border line between grades that counts need to be made. The examination, however, must be sufficiently thorough to make sure of the grade as specified above.

When the microscopic method is used as a rapid method of estimating the standard plate count, the count should be based on the number of groups of bacteria present, isolated individual bacteria being counted as one group. Such a group or clump count can, with experience, be made to correspond fairly accurately with the plate count. Accurate counting, however, involves the individual judgment to such an extent that skill can only be obtained with practice and by continual comparison of the group count with the standard plate count.

In order to insure careful work in grading, it is required that laboratories conforming to standard procedure shall preserve microscopic preparations until a reasonable period has elapsed after the reports are rendered to the person or persons whose milk has been examined. It is an excellent custom occasionally to have the grad-

ing done by one analyst and then repeated by a second analyst, particularly in those cases where punitive actions are to be based on the reports made.

COMMON SOURCES OF ERROR IN COUNTS

Routine microscopic counts, like all bacterial counts, are to be regarded as estimates of numbers only. They cannot be made with absolute accuracy even with the most careful technique. Errors will arise from inaccuracies in measurement of the minute quantities of milk examined at any one time, from faulty staining or preparation of slides, from mistakes in observation, and the like. These limitations, while important, are not difficult to overcome in sufficient measure to make microscopic grading a satisfactory method of controlling the quality of unpasteurized milk. As it is only in this way that counts of the bacteria themselves can be made, it must be recognized that accurately carried-out microscopic counts of individual bacteria give the truest picture of the actual conditions of raw milk that can be obtained with any technique.

Where there is reason to expect the presence of dead as well as living organisms — as, for example, in pasteurized milk — it is improper to regard microscopic counts as counts of living bacteria. Valuable information may, however, be obtained by making comparative plate and microscopic counts from samples of pasteurized milk.

REPORTS

As only a few ordinances have yet been adopted in which both plate and microscopic count standards have been given, the form of report used will need to be adapted to the circumstances under which each laboratory is working. Specific counts should not ~~be~~ given under normal circumstances, and care should be taken to avoid making finer distinctions in grade than are justified by the accuracy of the grading. A series of samples should be examined in all cases before rendering judgment as to the quality of any milk supply.

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